RNA expression in sperm as markers of sperm-quality

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Abstract

In the last decades, an apparent decline in sperm-quality has been observed in the western world. The reasons for this development are largely unknown, but environmental agents may play a role. Polycyclic aromatic hydrocarbons (PAH) are widespread toxicants known to negatively affect reproductive health, and have been shown to damage sperm DNA, by forming bulky DNA adducts and oxidative DNA damage. An important cause of poor sperm-quality is believed to be sperm DNA damage, which is linked to pregnancy loss after assisted fertilization, disturbances in embryo development and increased risk of childhood cancer. Obviously, the sperm DNA integrity seems to be important for fertilization and early embryo development, therefore, finding sperm-quality markers would help to understand the causes of male infertility and to improve the male reproductive health. Sperm contain a complex population of RNA, and the utility of sperm RNA in fertility research are currently being explored. In this study, the aim was to examine if RNA can be used as sperm-quality markers, by investigating the expression of some selected sperm mRNA and miRNA species after exposing mice to Benzo[a]pyrene (BaP), commonly used as model for exposure to PAH. Testis and liver was included for comparison. Importantly, we wanted to establish a procedure to isolate good quality RNA from individual sperm samples. The mRNA and miRNA expression was analyzed 24 days after exposure using real-time quantitative PCR (qPCR). The RNA isolation procedure was successfully established, but there is a need for the future inclusion of a marker of potential somatic cell contamination. Our results showed no effect of BaP on gene expression in liver, however, a differential expression of a few genes in testis and sperm was indicated. To our surprise, we observed a marked effect of vehicle exposure on gene expression in all three tissues examined. Interestingly, the ratio of the *protamine1* and *protamine2* genes was slightly altered in testis, and markedly changed in sperm, in response to both corn oil- and BaP-exposure (not significant). The protamine ratio is believed to be associated with sperm-quality in humans. The miRNA expression data are preliminary, but we did succeed in the identification of the selected transcripts in sperm. The findings suggested that RNA expression can be reliably analysed using sperm isolated from single mice. This makes RNA expression studies attractive for both experimental and clinical studies. We showed some significant effects on gene expression in sperm from BaP and/or corn oil exposed mice. Corn oil has previously been shown to induce and potentiate oxidative damage in sperm which could thus be an explanation of the responses seen in the present study. The *prm1:prm2* ratio in testis and sperm may be used as predictive sperm-quality markers, but further validation is needed. Furthermore, substitutes for corn oil as vehicle should be considered when investigating BaP-induced gene expression changes.

Abbreviations

ANOVA	Analysis of Variance
Apex1	Mammalian AP Endonuclease 1
ATSDR	Toxicological profile for polycyclic aromatic hydrocarbons
BaP	Benzo[a]pyrene
BPDE	B[a]P-7,8-diol-9,10-epoxide
Bw	Bodyweight
cDNA	Complementary DNA
Crem	cAMP responsive element modulator
CYP	Cytochrom
Cyp1a1	Cytochrome P450, family 1, subfamily A, polypeptide 1
Cyp1b1	Cytochrome P450, family 1, subfamily B, polypeptide 1
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside Triphosphate
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
miRNA	MicroRNA
mRNA	Messenger RNA
OGG1	8-OxoGuanine Glycosylase1
PAH	Polycyclic aromatic hydrocarbons
PCR	Polymerase chain reaction
Prm1	Protamine 1
Prm2	Protamine 2
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT (enzyme)	Reverse Transcriptase
RT-qPCR	Real time quantitative PCR
SCLB	Somatic cell lysis buffer
SE	Standard Error

tRNA	transfer RNA
WHO	World health organization
WT	Wild type
Xpc	Xeroderma pigmentosum, complementation group C
XRCC1	X-ray Cross Complementing Protein 1
PBS	Phosphate buffer solution

1 Introduction

1.1 General background

Negative trends on male reproductive health have been observed in western countries in the last decades, as revealed by the increased incidence of testicular cancer, genital abnormalities, poor sperm-quality and sub-fertility (reduced fertility). These male reproductive disorders are believed to be related by the Testicular Dysgenesis Syndrome (TDS). The causes of this adverse development are not known, but exposure to an increasing number of environmental toxicants including cigarette smoke, is a growing concern (reviewed by (Boisen *et al.*, 2001)).

DNA damage in sperm is associated with poor sperm-quality (Fraga *et al.*, 1996; Irvine *et al.*, 2000; Ni *et al.*, 1997). Zenzes et al. (1999) showed that cigarette smoking caused DNA damage in sperm that were transferred to the early embryo. In addition, an association has been described between fetal tobacco exposure (exposed by mothers smoking while pregnant) and a lower sperm-quality (Jensen *et al.*, 2005). In recent years, there has been an increase in assisted fertilization, and about half of the cases are related to male infertility. One of 80 born children in the US, one of 40 born in Australia and one of 24 born in Denmark are conceived using assisted fertilization (reviewed by (Aitken *et al.*, 2009)). There is growing evidence that sperm DNA damage is related to pregnancy loss after assisted fertilization (Aitken *et al.*, 2009; Badouard *et al.*, 2008; Macklon *et al.*, 2002), as well as disturbances in embryo development and mutation in the progeny (Ahmadi and Ng 1999; Macklon *et al.*, 2002). This indicates that the DNA integrity of the sperm is critical both for male fertility and for embryo development. To help improve the reproductive health, developing markers for sperm-quality of relevance for male fertility and the health of the offspring, would be helpful. It would also allow for an increased understanding of causes and mechanisms related to the reduced male fertility. Sperm cells are easy accessible and the utility of sperm cell analyses for assessing male infertility makes them attractive tools.

The adverse trend of male reproductive disorders has occurred within a relatively short period of time, suggesting that environmental factors may be of importance (reviewed by (Moline *et al.*, 2000). The polycyclic aromatic hydrocarbons (PAHs) are widespread environmental agents suspected to negatively affect male reproductive tissue (ATSDR 1995).

Benzo[a]pyrene (BaP) is a well-studied PAH that can be used as a marker of exposure to, and effect of, the carcinogenic and genotoxic PAHs, which was concluded by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (EFSA 2008). BaP is a commonly used model for PAH exposure to study the effect on male reproductive tissue, such as induction of DNA adducts in sperm and testis cells of mice, but also morphological and endocrine disrupting effects (Archibong *et al.*, 2008; Mohamed *et al.*, 2010; Olsen *et al.*, 2010; Verhofstad *et al.*, 2010a)

The mature sperm cell retains a complex RNA population, including the cellular regulators which silence or suppress gene expression, microRNAs (miRNAs) (Ostermeier *et al.*, 2005b). The utility of sperm RNA as markers for infertility has been explored (Miller 2000a; Miller 2000b; Steger *et al.*, 2001) where differences in transcript levels in sperm of different motility (Lambard *et al.*, 2004), as well as between normal and abnormal sperm samples (Platts *et al.*, 2007; Steger *et al.*, 2003), have been reported. All these studies tried to correlate the mRNA transcript levels with sperm quality and infertility. In this study, the suitability of sperm RNA as possible markers for sperm-quality was investigated together with exposure to the PAH model compound, BaP.

1.1.1 Aims

The overall aim of this study was to examine whether sperm RNAs can be used as novel sperm-quality markers. In order to do this, we investigated sperm mRNA and miRNA expression of some selected genes after exposing mice to BaP.

The specific aims were:

- Establishing a procedure to isolate good quality sperm RNA from a single mouse, by developing a methods to:
    - Isolate sperm cells without contamination of somatic cells
    - Extracting the small amount of RNA present in sperm

- Identifying BaP-induced changes in the expression of some selected mRNAs and miRNAs in sperm. Testis and liver tissues were included for comparison.

1.2 PAH and benzo[a]pyrene

1.2.1 Sources and occurrences

BaP is an archetypical PAH released into the environment as a result of forest fires, industrial emissions, fabrication of products such as tar, coke and aluminum, automobile exhaust and spill of oils from tankers. Release into the residential environment is caused by combustion of coal and wood, including cigarette smoke (ATSDR 1995). Humans are exposed to PAHs through several pathways, but for non-smokers the major route of exposure in through food ingestion. Food can be contaminated with PAHs from environmental sources, industrial food processing and from specific home cooking practices, for example from barbecuing food (EFSA2008).

In the Nordic countries, BaP in ambient air is rarely expected to exceed 1.0 ng/m^3. In a highly trafficked area in Oslo (2002/2003), the BaP concentration was measured to be between 0.2 and 1.6 ng/m^3. The intake of BaP via inhalation, assuming a ventilation volume of 20 m^3 per day, is expected to be between 4 and 32 ng per person (EFSA 2008).

Smokers are substantially more exposed to PAHs and BaP. The content of BaP in commercially available filter cigarettes in Spain, UK and USA, was measured to be in the range from 2 – 20 ng per cigarette. A person smoking 20 cigarettes a day, assuming 6.5 mean delivery and 80 % of inhaled particle bound is distributed in the respiratory tract, the intake is 105 ng (EFSA 2008).

1.2.2 Metabolism and toxicity

BaP is classified as a carcinogen in category 1B (presumed to have carcinogenic potential for humans) within the European Union, according to the Classification, Labelling and Packaging regulations (CLP). BaP is also classified as toxic to reproduction, category 1B (may damage fertility; may damage the unborn child).

When BaP enters the body, it is metabolized by phase I and phase II enzymes with the purpose of detoxification and elimination. In the initial stage of BaP metabolism, the phase I enzymes; epoxide reductases, epoxide hydrolase, and cytochrome P450s (CYP450) mixed function oxidases, give rise to hydrophilic metabolites. The intermediate is then further metabolized to more polar products by the phase II conjugating enzymes; such as glutathione transferases, UDP-glucuronyl transferases and sulfotransferases. Conjugation of BaP results in formation of products that is more hydrophilic than the parent compound that can be excreted from the body. In addition to detoxification of BaP to harmless hydrophilic compounds, the CYP450s and epoxide hydrolase can convert BaP to highly mutagenic and carcinogenic metabolites, such as B[a]P-7,8-diol-9,10-epoxide (BPDE), that are reactive towards DNA and are designated as the ultimate carcinogen (figure 1-1) (reviewed by (Miller and Ramos 2001; Shimada 2006))

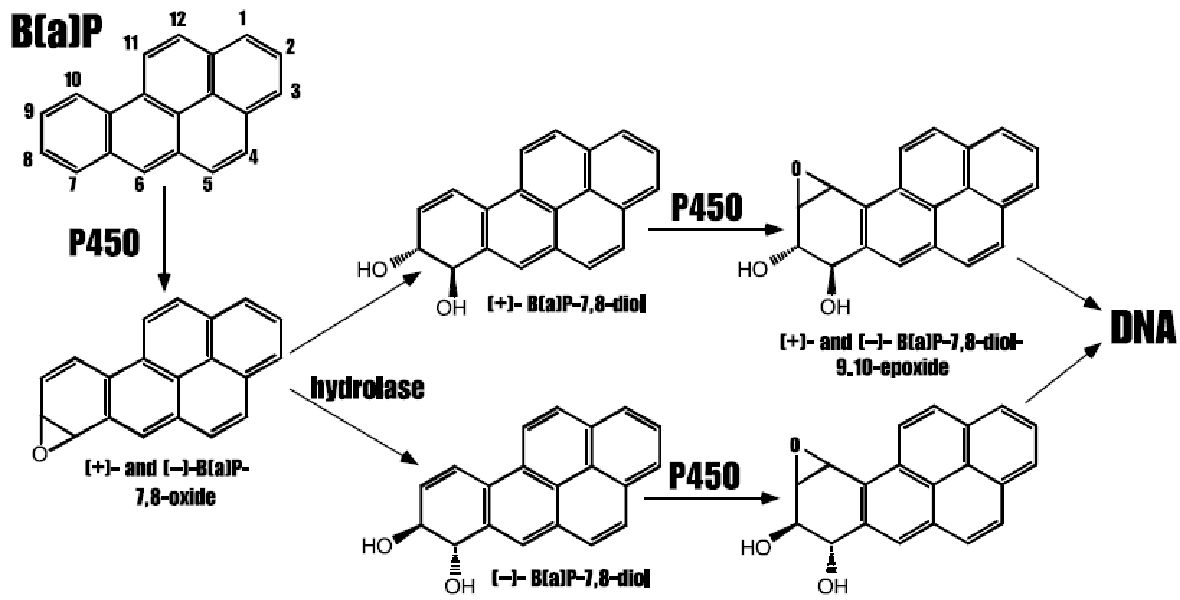


Figure 1-1: Metabolic activation of BaP *by the Phase I enzymes CYP450 generating the ultimate carcinogen, B[a]P-7,8-diol-9,10-epoxide (BPDE), that are reactive towards DNA (Shimada and Fujii-Kuriyama 2004).*

The CYP enzymes, cyp1a1 and cyp1b1 have key roles in activating BaP to the harmful metabolite BPDE. They are induced by BaP via the aryl hydrocarbon receptor (AhR) which starts a cascade of reactions leading to transcription of the *cyp1a1* and *cyp1b1*. After protein synthesis, both enzymes are involved in the activation of BaP to form (-) B(a)P-7,8 dihydrodiol, which is then further oxidized by epoxide hydrolase to give BPDE (Shimada 2006; Shimada and Fujii-Kuriyama2004)

The BPDE metabolite, including a various of other BaP metabolites, can cause DNA damage in the form of DNA adduct, as their metabolites bind covalently to deoxyadenosines and deoxyguanosines (WHO 1998). Usually these DNA adducts are removed by DNA repair mechanisms within the cell nucleus, but unrepaired DNA damage can give rise to DNA mutations and can lead to the growth of malignant tumors (Miller and Ramos 2001).

BaP is a well-known reproductive toxicant, and have been associated with chromosomal replication error and DNA damage in gametes, altered sperm morphology and decreased sperm numbers (ATSDR 1995; Mohamed *et al.*, 2010; Olsen *et al.*, 2010; Verhofstad *et al.*, 2010a) and egg numbers (Zenzes *et al.*, 1998). High levels of acute exposure have been reported to be associated with immune system suppression and red blood cell damage, which can result in anemia (ATSDR 1995). The oxidative metabolism of BaP can also adversely affect the embryo. Studies have reported resorption and malformation of fetuses (Legraverend *et al.*, 1984) and sterility of mouse progeny whose mother have been exposed to BaP (Mackenzie and Angevine 1981). It has also been demonstrated that paternal exposure to BaP has the ability to be transferred, via sperm, to the next generation. Mohamed *et al.* (2010) reported a decrease in sperm count and reduced fertility in mice progeny of paternally BaP-exposed mice. In a dominant lethality study in mice, BaP-exposure in 1-2 weeks preceding fertilization induced increased rate of embryo losses (Generoso *et al.*, 1982).

1.3 Male germ cells

1.3.1 Spermatogenesis and BaP exposure

Spermatogenesis is the process in which spermatogonia form spermatozoa, and is essentially similar in all mammals. The course of action may be divided into three phases based on functional considerations:

1. The proliferative phase – The spermatogonium undergo mitosis.
2. The meiotic phase – The primary spermatocyte undergo meiosis I to yield two secondary spermatocytes, which again go through meiosis to become spermatids.
3. The differentiation or spermiogenic phase – Spermatids differentiate to become spermatozoa, which are equipped to reach and fertilize the egg (Adler 1996).

The purpose of the spermatogenesis is to build and maintain a population of fully differentiated spermatozoa, which most mammalian species can produce millions of per day.

To achieve this, the cell population is increased at an early stage beginning with the relatively immature spermatogonial cells which are located to the base of the seminiferous epithelium. There are three types of spermatogonial cells; stem cell spermatogonia, proliferative spermatogonia and differentiating spermatogonia. It is the differentiating spermatogonia that divide mitotically to form primary spermatocytes, which is the first cell of the meiotic phase (Adler 1996; Hess 1998; Russell *et al.*, 1990).

The meiotic phase lasts 25 days in humans and 14 days in mice, with the first meiotic division occupying the majority of the time. During the prophase of the first meiotic division, DNA synthesis and further condensation of the DNA occurs and involves numerous types of spermatocytes that range in sizes. The end product of meiosis I is small secondary spermatocytes consisting of one pair of chromosomes (2N). The duration from the end of the prophase to the second meiotic division, is only less than 24 hours. The chromatids become separated and move to opposite spindle poles, to eventually form small haploid cells (1N) called round spermatids (reviewed by (Hess1998; Marchetti and Wyrobek 2005)). During the differentiation, also known as spermiogenesis, the round spermatid undergoes a series of changes where the acrosome and the flagellum develops, the nuclei condensate and cytoplasmic constituents are lost. At this point they have become spermatozoa that are ready for release into the seminiferous tubular lumen for transport to the seminal vesicle (Holstein *et al.*, 2003; Russell *et al.*, 1990).

In our study, the sperm cells were collected from cauda epididymis 24 days after BaP-exposure. Sperm cells entering the epididymis after 24 days, would have been in late spermatocyte stage at the time of exposure (figure 1-2). At this stage, the nuclear chromatin is still not condensed, which means that BaP is able to induce a transcriptional DNA damage response. If DNA damage was induced at an earlier stage, they would more likely be removed due to the DNA repair capacity of the spermatogonia and spermatocytes. This capacity to repair the damaged DNA is lost shortly after they become spermatids (Olsen *et al.*, 2005). Thus, in the presence of BaP induced DNA damage, we wanted to investigate possible molecular changes in mature sperm cells in the search for a novel sperm-quality marker.

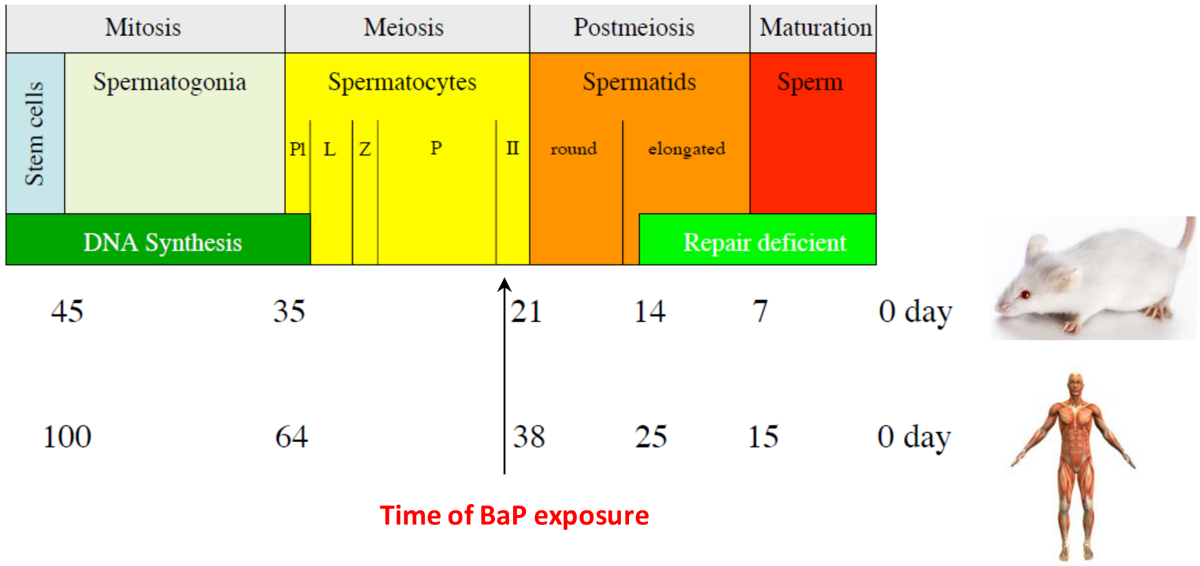


Figure 1-2 Schematic illustration of the mammalian spermatogenesis. *The duration of the spermatogenesis for mice and men are shown along with the different stages of the spermatogenesis. Approximate duration of the DNA synthesis and DNA repair deficiency are indicated. Pl: preleptotene; L: leptotene; Z: zygotene; P: pachytene; III: meiosis II (Marchetti and Wyrobek2005).*

1.3.1.1 Sperm RNA

RNA in mature sperm cells was first assumed to be lost or degraded during the extensive cellular modifications that spermatids undergo during spermiogenesis. Thus, detection of a complex and fully intact mRNA population started a debate regarding the possible function of the sperm RNAs. Until recently, the RNA in sperm was believed to be non-functional, but several experiments have shown that RNA might perform active functions both within the cell itself and perhaps once it gains access to the oocyte after fertilization (Miller *et al.*, 2005; Miller 2011)

It is debated whether sperm are translationally silent, because studies have shown that it does not have sufficient 80S cytoplasmic ribosomal complexes to support this process. There are reports suggesting that sperm RNA is translated on the mitochondrial machinery, but it still remains unclear how mRNA marked with 5’ recognition sequences designed for 80S ribosomes are translated in mitochondria (reviewed by (Galeraud-Denis *et al.*, 2007; Miller and Ostermeier 2006)).

Sperm RNA may also play a role in the embryo development. Ostermeier *et al.* (2004) demonstrated that sperm RNA is delivered to the oocyte, because sperm RNA was detected in the zygote after fertilization. Rassoulzadegans *et al.* (2006) provided a model for epigenetic inheritance by zygotic transfer of RNAs that dysregulate expression of the so-called *c-Kit* gene, which leads to the modification of phenotypic expression of the offspring. This model showed that the spermatozoa can affect development processes by operating independently of the paternal genome.

In the field of infertility research, there are a variety of tests available which assess sperm-quality and function. Thus, there is still a need for better and more reliable methods when considering that male factor infertility is involved in at least 50 % of unexplained infertility cases (Hwang *et al.*, 2011). Some methods developed for analyzing sperm-quality are presented below, followed by infertility research sperm RNA.

1.4 Sperm-quality assays

Semen analysis is the most important diagnostic tool used to assess fertility (Hwang *et al.*, 2011). The World Health Organization (WHO) have established reference values for normal semen parameters such as sperm count, morphology, motility and viability which are used as diagnostic tools for physicians when evaluation patients with fertility problems in the clinic (WHO 1998). However, neither alone nor in combination are these guidelines effective predictors of infertility (Lalancette *et al.*, 2009).

Additional methods evaluating the sperm's function have been developed to provide clarification include the osmotic swelling test (Jeyendran *et al.*, 1984), the sperm penetration assay (Carrell 2000) and the sperm structural assay (Evenson *et al.*, 1999). Other assays use the quantification of harmful reactive oxygen species (ROS) to evaluate sperm-quality. An excess of ROS can lead to sperm damage and infertility; ROS levels are elevated in up to 40% of subfertile men (Iwasaki and Gagnon 1992). ROS can also be generated during the metabolism of BaP (Miller and Ramos 2001). Assays for evaluating the presence of sperm DNA damage has also been developed, and sperm DNA damage is associated with poor semen quality (Fraga *et al.*, 1996; Irvine *et al.*, 2000; Ni *et al.*, 1997). There are several methods available; the comet assay, the acridine orange–staining test and the terminal deoxynucleotidyl transferase mediated nick-end labeling (TuNEL) test. However, in spite of the various sperm tests, a significant percentage of male-factor infertility remains unexplained.

1.4.1 The utility of sperm RNA as sperm-quality markers

Methods to detect submicroscopic genetic abnormalities are emerging, providing molecular data about the underlying biochemical mechanisms of idiopathic infertility. The use of sperm RNA to predict fertility and sperm-quality has been investigated, and several studies have characterized and compared sperm RNAs between fertile and infertile men. An interesting finding is the presence of different transcript levels in sperm of different motility (Lambard *et al.*, 2004; Platts *et al.*, 2007; Steger *et al.*, 2003; Steger *et al.*, 2008), as well as between normal and abnormal sperm samples. Lambard *et al.* (2004) showed that the amount of *prm1* transcripts in the poorly motile subpopulation of sperm was higher compared to cells obtained from a population with higher motility. Similar findings were done by Wang *et al.* (2004) where quantitative sperm mRNA changes were related to the motility of the sampled population. Steger *et al.* (2008) found an altered ratio of *prm1*:*prm2* transcripts in sperm between infertile and fertile men; 1:1.7 versus 1:1, and suggested that these genes could be a useful marker for predicting male infertility.

The transcripts *prm1*, together with *prm2*, codes for protamines which are responsible for packaging the sperm chromatin tightly and is important for protecting the paternal genome (Carrell *et al.*, 2007). The protamines 1 and 2 have been studied extensively in relation to male infertility, and an altered protmaine1:protamine2 ratio at the protein level has been associated with reduced male fertility (Oliva 2006).

Sperm transcripts may originate from earlier stages of spermatogenesis, and reflect previous testicular events (Ostermeier *et al.*, 2005a). The findings of different spermatozoa transcripts in different sperm population related to different motility, could provide information about the microenvironments of the testis that give rise to these sperm subpopulations. It could give insight of how these environments may be affected by pathological and environmental disturbances. Because environmental agents are believed to affect male fertility and sperm-quality, sperm RNA may be the best opportunity we have for understanding these effects at the molecular level (Miller and Ostermeier 2006).

1.5 MicroRNA

MicroRNAs (miRNAs) are short nucleic acids, ~22 nucleotides in length, and important regulators of gene expression in mammals. They regulate gene expression by binding target mRNAs and inhibit translation, by either sequestration or degradation. MiRNAs are widely expressed in all tissues and developmental stages, and ~30 % of all human genes are predicted to be regulated by miRNAs (reviewed by (Hudder and Novak 2008)).

The impact of environmental exposure has on miRNA expression has not been given much attention. Some studies have investigated the effect of exposure to toxicants, such as BaP and dioxin in rodent liver (Moffat *et al.*, 2007; Yauk *et al.*, 2011), but limited response was detected. Even though minor changes were seen, changes in miRNA expression less than twofold may have considerable biological effects due to the large number of potential targets regulated by each miRNAs (Calin *et al.*, 2002).

MiRNAs have also been shown to play a key role in epigenetic regulations. It is believed that changes in miRNA expression in response to chemical exposure *in utero*, may result in developmental abnormalities or oncogenesis (reviewed by (Hudder and Novak 2008)). Paternal influence on offspring development have received increased attention, and there is evidence that RNAs, including miRNAs, of sperm cells can carry functional epigenetic information that can be inherited trangenerationally through the germline (Rassoulzadegan *et al.*, 2006).

The extensive role of miRNAs in various developmental processes, make it a highly interesting candidate marker for chemical exposure and a potential marker for sperm-quality.

1.6 The use of mouse models

The study of reproductive toxicity often uses a rodent model to gather information about potential hazards, dose response and critical threshold for fertility (Moline *et al.*, 2000). In this study, the main focus was to investigate BaP-induced gene expression in sperm from mice to evaluate if a sperm-quality marker can be developed for humans. The use of a mouse model was done to establish the procedures for examining the gene expression in sperm in response to BaP exposure. Furthermore, the findings in this study may show whether the expression of sperm RNAs are influenced by chemical exposures. For this purpose, the use of mouse models for comparison to humans is valuable.

2 Materials and methods

All solutions and chemicals used in this experiment are listed in appendix B.

2.1 Mice

2.1.1 Breeding and care

In the BaP experiment, C57BL/6J BomTac male mice were purchased from Taconic (Ejby, Denmark). The mice acclimatised for a week after arrival. C57BL/6 mice used for establishing the RNA isolation procedure were bred by the Norwegian Institute of Public Health (NIPH), Oslo, Norway. Animals were housed in air flow IVC racks (Thoren Maxi-Miser System) and filter cabinets (Scantainer, Scanbur BK AS, Nittedal, Norway) in plastic disposable cages on Nestpack (Datasand Ltd., Manchester, UK) bedding. They were exposed to a 12 hour light/dark cycle, 6-10 air changes per hour, controlled humidity (55±5%) and temperature (19-23°C). Water and diet were given *ad libitum*. The mice were given a maintenance diet, SDS RM1 (Special Diet Services, Witham, U.K). All research was performed in conformity with the laws and regulations for experiments with live animals in Norway.

2.1.2 Harvesting of organs

The mice were sacrificed by cervical dislocation. The organs harvested were liver, testes, cauda epididymis and vas deferens. After removing the testicular capsule, the testes and a piece of the liver were immediately frozen on dry ice, and stored at -80°C. Cauda and vas deferens were kept in Hepes buffered medium (M2 medium) and placed on ice before collecting the sperm.

2.2 Exposure of mice to BaP

2.2.1 Dissolving BaP in corn oil

BaP is lipophilic and is commonly dissolved in corn oil. It is a carcinogen and must be handled with great care. Ventilation cabinets were used when dissolving BaP in corn oil.

The procedure:

1. BaP was weighed in a glass vial, corn oil was added (at room temperature) to obtain a stock solution of 7.5 mg BaP/ml corn oil.
2. The bottle was placed in a shaking water bath at 37º C for 1 hour to dissolve.
3. A magnetic stirrer was used to dissolve the remaining unsolved BaP.
4. The bottle was covered with aluminium foil and stored in a dark, dry and ventilated security cabinet at room temperature.

2.2.2 Exposure of mice

Ten male mice (8 weeks of age) were injected intraperitoneally with 50 mg/kg bodyweight (bw) BaP dissolved in corn oil on three following days (day 0-2). A total amount of 150 mg/kg bw BaP was given to each mouse. The dose of BaP injected was based on a previous study performed by (Olsen *et al.*, 2010). Six mice were exposed to corn oil (vehicle control) and four untreated mice were used as controls. The first day of exposure was defined as day 0, and sacrifice was conducted at day 24. Mice exposed to the same treatment were kept together in cages two and two, and individually marked by ear punching.

2.3 Isolation, purification and counting of sperm

2.3.1 Isolation of sperm cells

It is important to eliminate somatic cells when collecting the sperm to obtain pure sperm for down-stream analysis such as sperm specific mRNA expression. A somatic cell contains ~600-fold more mRNA compared to a sperm cell and should therefore be avoided (Galeraud-Denis *et al.*, 2007). Fat tissue and the major blood vessel present on vas deferens were removed to reduce somatic cell contamination. In addition, the sperm-containing solution was filtered to remove tissue fragments. Sperm were collected from cauda epididymis and vas deferens soon after sacrifice.

The procedure was performed on a cold metal plate to avoid RNA degradation.

Procedure:

1. Cauda and vas deferens from one mouse were dissected and transferred to a small tissue culture dish containing 1 ml of M2 medium. Fat tissue was removed using micro scissor and tweezer under a stereo microscope (Olympus SZX16).
2. Cauda and vas deferens were rinsed in phosphate buffered saline (PBS) and placed on a new culture dish with 1 ml of M2 medium.
3. To release the spermatozoa, an opening was made in cauda and vas deferens by using a micro scissor. A bended needle was used to gently squeeze the cells into medium.
4. Sperm from one mouse (two cauda and two vas deferens) were collected and filtered (100 µm nylon filter) to avoid tissue fragments. The cell solution was then diluted in PBS to the volume of 1.5 ml in an eppendorf tube.
5. Samples were centrifuged at 600 x g for 5 minutes at 4° C. Supernatant was removed.

2.3.2 Purification of sperm-solution by somatic cell lysis

To remove remaining somatic cells, the spermatozoa-containing solution was treated with a hypotonic buffer; somatic cell lysis buffer (SCLB; 0.1 % SDS, 0.5 % Triton X;100 % solved in deionised water). This procedure was based on the method previously described by (Goodrich *et al.*, 2007).

Procedure:

1. 1 ml SCLB was added to the sperm pellet and resuspended by pipetting, and then transferred to a small falcon tube.
2. Another 4 ml SCLB was added before incubation on ice for 30 minutes.
3. At 20 minutes a sample from the SCLB solution was pipetted onto an object glass and inspected under a microscope to verify the absence of somatic cells. If present, the sample was centrifuged at 200 x g for 15 minutes at 4° C, and step 1-3 was repeated. If absent, step 4 was proceeded.

4. The sample was centrifuged at 600 x g for 15 minutes at 4° C. Supernatant was removed.
5. To remove traces of SCLB, the sample was washed twice, each time with 1 ml PBS and lastly centrifuged at 600 x g for 5 minutes at 4° C.

2.3.3 Counting of sperm cells

Bürker counting chamber (Labor Optik®, Bad Homburg, Germany) was used to determine the concentration of sperm and contaminating somatic cells (e.g white blood cells, fat cells). An imprinted grid on the chamber surface (figure 2-2A) allowed for an easy counting of cells in a known volume. The grid is divided into 9 large squares, and each square is bounded by a triple set of lines. The center line of these is the edge of the counting area. All the sperm heads/somatic cells within the central lines of a large square were counted, also those which touched the upper and right border, but not those which touch the lower and left borders (figure 2-2B).

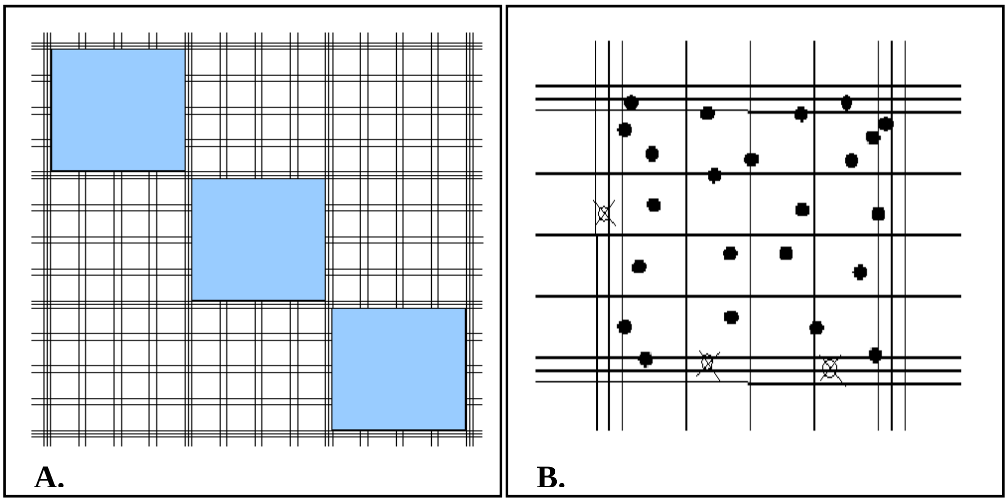


Figure 2-2 A. The full grid of the Bürker counting chamber. *The grid has 9 large squares, the blue squares show the counting area if the number of cells were between 50-200.* **Figure 2-2 B. One of nine large squares within the grid of the Bürker counting chamber.** *Cells that touch the left and the lower central line should not be counted (marked with X).*

The sperm were diluted into distilled water with the intention to immobilize the sperm and to make sure that the cells did not overlap each other on the grid. The dilution did vary depending on the concentration of the sperm-containing solution. To determine a correct estimate of the cell concentration, the sample had to be thoroughly mixed before adding the diluting fluid, and before loading the chamber.

Procedure:

1. The cover glass and the counting chamber was dried off with a lens paper.
2. The cover glass was placed on the counting chamber after moistening the rails with distilled water. This prevented the cover glass from moving when loading the sperm sample.
3. 10 µl of diluted sperm was pipetted under the cover glass, and the cells were counted under a microscope with a 10 X objective.
4. At least 150 sperm should be counted (in each grid) before calculating the concentration. Number of squares to be counted are determined by the number of sperm in the one square; < 50 – count all the 9 squares; 50-200 – count 3 diagonal squares; >200 – the solution should be further diluted.
5. Loose sperm heads were included in the count, and somatic cells were counted separately.

2.3.3.1 Calculation of cell concentration

The chamber was 0.1 mm deep and one large square represented an area of 1 mm^2. Thus, the volume of fluid above one large square is (0.1) x (1.0) = 0.1 mm^3 or 0.1 µl. In this way, the concentration of the original sperm sample can easily be estimated.

Procedure:

1. The mean number of sperm of one large square was calculated.
2. The mean count of one square was multiplied with the volume above one square to find the number of cells in the diluted sperm sample.
3. To obtain the concentration of the original sperm sample the dilution factor must be multiplied. Final equation:

   <u>Cells pr ml in the original sample = mean count in one square * dilution factor * volume</u>

2.4 Gene expression analysis by real-time quantitative PCR (RT-qPCR)

To investigate changes in miRNA and mRNA expression level followed by BaP-exposure, gene expression analysis was carried out using RT-qPCR. This method enables the expression of selected genes to be studied. In order to do this, total RNA was extracted from the control and BaP-exposed tissues and reversed transcribed to complementary DNA (cDNA) and qPCR was performed.

2.4.1 RNA isolation

Total RNA was isolated from sperm cells, liver and testis, from BaP-exposed mice, vehicle controls and untreated controls. Tissue was prepared as described in section 2.1.2 (liver and testes) and 2.3 (sperm).

The RNA isolation was performed using miRNeasy Mini kit (Qiagen, Germany), according to manufacturer's instructions, with some modifications; the tissue was homogenized by Ultra turrax (IKA®-Werke, Staufen, Germany), a tool designed to lyse and homogenize biological samples, with blades that rotates at high speed. It causes the sample to be disrupted by a combination of turbulence and mechanical shearing.

RNA is easily degraded due to the wide variety of RNases in the environment. Hands and dust particles may contain bacteria and molds, which are the most common sources of RNases. Great care should be taken when handling RNA samples, both during and after the isolation procedure to avoid unwanted changes in the expression profile. Benches and all involved equipment were cleaned with RNase-Away, an RNase inhibitor, to prevent RNase contamination. When starting the isolation procedure, it is very important not to allow the tissue to thaw before coming in contact with the lysis solution (QIAzol) from miRNeasy mini kit. The lysis solution contains RNase inhibitors and prevents RNA degradation (miRNeasy Mini Handbook 2007).

The small amount of RNA present in sperm makes it very challenging to extract sufficient amount of RNA suitable for downstream analysis. Hence, addition of carrier RNA during sperm RNA extraction is recommended in order to increase the recovery of total RNA. Carrier RNA also prevents that the small amount of target nucleic acid present in the sample from being permanently bound to the silica membrane of the RNA extraction spin column. To test the effects of using a carrier, total RNA was isolated with and without carrier RNA and the results was compared. Yeast tRNA was used as a carrier.

RNA isolation procedure (illustrated in figure 2-3)**:**

1) Preparation

a. Preparation of tissue

Liver and testis:

The frozen piece of tissue was weighed and transferred to a tube containing 700 µl of QIAzol lysis. If more than 20 mg of tissue was used, equally more QIAzol lysis was added. The lysis solution facilitates lysis of the tissue (or cells), inhibits RNases and removes cellular DNA and proteins by initiating phase separation (see step c).

Sperm:

Immediately after the somatic cells were lysed, 700 µl of QIAzol lysis solution was added to the sperm pellet. The lysis solution volume was adjusted according to the predicted mean number of sperm (the prediction was based on previous sperm counts).

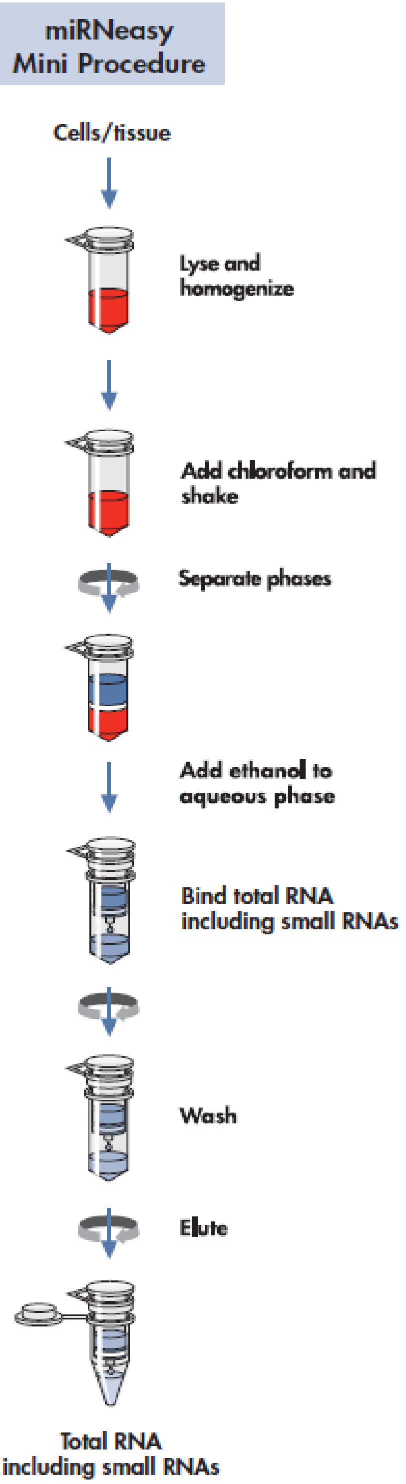


Figure 2-3. Illustration of the RNA isolation procedure. *Initially, the cells/tissue was lysed and homogenized (step 1b). After adding chloroform (step 1c) the solution separated into 3 phases. Then the aqueous phase mixed with ethanol was transferred to a spin column, where the total RNA (mRNA including miRNA) bound to the membrane. Three washes were performed to remove contamination, and lastly the RNA was eluted in RNase-free water (miRNeasy Mini Handbook 2007).*

b. Homogenization of tissue:

After adding the lysis solution, homogenization by using Ultra-turrax was performed for 1 minute, or until no clumps are visible.

c. Phase separation:

i. The tube containing the homogenized sample was placed on the benchtop at room temperature (25° C) for 5 minutes to promote dissociation of nucleoprotein complexes.

ii. 140 µl of chloroform was added to the homogenate and vortexed for 15 seconds. This was important to induce phase separation. The volume of chloroform was also adjusted to the amount of tissue or to the number of sperm, using the same procedure as described in step 1a.

iii. The tube was placed on the benchtop at room temperature for 2-3 minutes. At this time the separation of the solutions became visible.

iv. The sample was centrifuged at 12000 x g at 4° C for 15 minutes. After centrifugation, the sample separated into three phases, one upper colourless, aqueous phase containing the RNA; a white interphase containing the DNA; and a lower red organic phase containing proteins.

v. The upper aqueous phase was transferred to a new collection tube, about 350 µl. Care was taken not to aspirate the white material deposited on the tube wall within the protein-containing white interphase.

- 10 µl of a stock solution with a concentration of 0.4 µg/µl yeast tRNA (purchased from invitrogen) was added to the sperm samples and thoroughly mixed.

d. Preparation for binding of RNA to column:

1.5 volume of 70 % ethanol was added, and mixed by pipetting up and down. The ethanol was added to provide appropriate binding conditions for all RNA molecules from 18 nucleotides and upwards. The next step was continued without delay.

2) RNA isolation

a. Loading onto columns:

i. 700 µl of the sample was pipetted onto a RNeasy spin column (included in the miRNeasy mini kit) that was placed in a 2 ml collection tube and centrifuged at 8000 x g for 15 seconds at room temperature. During centrifugation the total RNA binds to the column membrane. Therefore, the flow-through containing the QIAzol lysis, could be discarded.

ii. The remainder of the sample was added to the RNeasy spin column. It was centrifuged at same speed and time as described in previous step. The total RNA was now bound to the column membrane.

b. Column wash:

i. 700 µl of the washing Buffer RWT, supplied by miRNeasy mini kit, was added and centrifuged at 8000 x g for 15 seconds. The flow-through was discarded.

ii. 500 µl of another washing Buffer RPE, supplied by miRNeasy mini kit, was added and centrifuged at 8000 x g for 15 seconds. The flow-through was discarded. Buffer RWT and RPE are washing buffers removing phenols (remains from QIAzol lysis buffer), salts and other contaminants during the centrifugation.

iii. Another 500 µl of Buffer RPE was added, and centrifuged at 8000 x g for 2 minutes. This is to make sure that no ethanol is carried over during RNA elution.

c. Elution of RNA:

i. The RNeasy Mini column was transferred to a new 1.5 ml collection tube. Sperm samples: 40 µl of RNase-free water was added directly onto the membrane inside the column, and centrifuged at 8000 x g for 1 minute to elute the RNA.

ii. Liver and testis samples: 40 µl of RNase-free water was added as described for the sperm samples, then, another 40 µl was added and eluted into the same collection tube. Due to high RNA contents in both testis and liver, a second elution was necessary to reach the optimal RNA concentration and to keep the salt concentration at a minimum.

Isolated RNA was kept on ice until storage at -80° C.

2.4.2 Reverse Transcription PCR

RNA cannot serve as templates for real-time qPCR. Instead, RNA must be converted into cDNA in order to detect mRNA and miRNA transcripts. This can be performed by a reverse transcription reaction using the reverse transcriptase enzyme. The reverse transcription reaction should result in a cDNA population that reflects the original mRNA and miRNA population. Synthesizing cDNA for detection of miRNA and mRNA require two different approaches and two sets of kits. Total RNA isolated in the previous section was used to synthesize cDNA for detection of both miRNA and mRNA. The methods are described below (section 2.4.2.1 and 2.4.2.2).

2.4.2.1 cDNA synthesis for mRNA detection

Total RNA was reverse transcribed using High-Capacity cDNA Reverse Transcription Kits without RNase inhibitor (Applied Biosystems, CA, USA) according to the manufacturer instructions.

cDNA was synthesized using random primers supplied by the kit. The random primers included in the kit are hexamers, and priming will take place on several places in the RNA template, thus synthesizing multiple single-stranded cDNA (sscDNA) of different lengths (figure 2-4). In addition to primers and reverse transcriptase enzyme, the reaction needs a nucleotide mix (dNTPs) and a magnesium containing buffer ($MgCl_2$) which were all supplied in the kit.

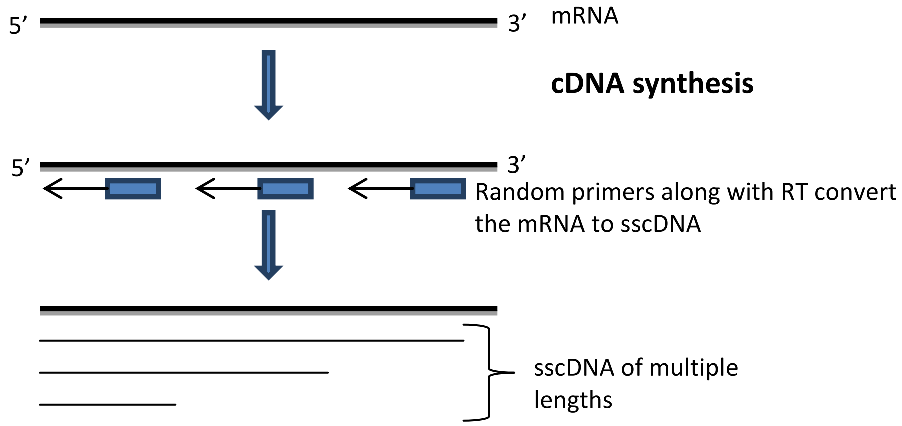


Figure 2-4. The reverse transcription reaction. *mRNA was reversed transcribed into cDNA using random primers that bound multiple sites at the mRNAs. These nonspecific primers provided a distribution of coding and non-coding sequences.*

To prevent DNA contamination when preparing for the reverse transcription reaction and real-time PCR, two separate hoods were used. Before use, 30 minutes exposure with UV-light was performed to destroy any DNA contamination. The master mix was prepared in the mastermix-hood and the RNA/cDNA samples were prepared in the DNA-hood.

Procedure:

Samples were kept on ice to avoid RNA degradation during the procedure.

1. Total RNA samples were diluted to contain 1 µg per 10 µl. Then 10 µl of the diluted RNA samples were pipetted to each well in a MicroAmp™ 96-well reaction plate (Applied Biosystems). The remaining RNA was stored at -80° C.
2. The master mix was prepared according to the Kit (table 2-1), and 10 µl was added to each well with the diluted RNA and mixed by pipetting up and down.

Table 2-1. Mastermix for cDNA reverse transcription PCR for mRNA detection.

Modified from Applied Biosystem High-Capacity cDNA Reverse Transcription Kits.

Components	Volume per well (ul)
10 x RT buffer	**2.0**
25 x dNTP Mix (100mM)	**0.8**
10 x RL random primers	**2.0**
MultiScribe™ Reverse Transcriptase	**1.0**
Nuclease-free H_2O	**4.2**
Total per reaction	**10.0**

3. The reaction plate was sealed with an optical adhesive cover (applied biosystems) and centrifuged at 1500 rounds per minute (rpm) for 1 minute to remove air bubbles and to spin down the content. The plate was placed on ice until loading the samples.
4. The cDNA synthesis was conducted using the thermal cycler (Eppendorf Mastercycler Gradient, Hamburg, DE), program shown in table 2-2.

Table 2-2. Thermal cycler program.

	Step 1	Step 2	Step 3	Step 4
Temperature	25° C	37° C	85° C	4° C
Time	10 minutes	120 minutes	5 minutes	∞

5. Quantification and purity of cDNA by NanoDrop 1000 Spectrophometer as described in section 2.4.3.
6. cDNA was stored at – 20° C until use.

2.4.2.2 cDNA synthesis for miRNA detection

MiRNAs were reversed transcribed using the miScript Reverse Transcription Kit (Qiagen, Germany).

The reverse transcription reaction was performed using oligo-dT, supplied in this kit. Unlike mRNAs, miRNAs are not polyadenylated. For this reason, the miRNAs were polyadenlyated with poly(A) polymerase. To detect miRNA during real time PCR analysis, the oligo-dT primers have a universal tag sequence on the 5' end which pair with a specific universal primer supplied by the real time PCR kit. The reverse transcription reaction followed by real-time qPCR using the miScript Kit is shown in figure 2-5.

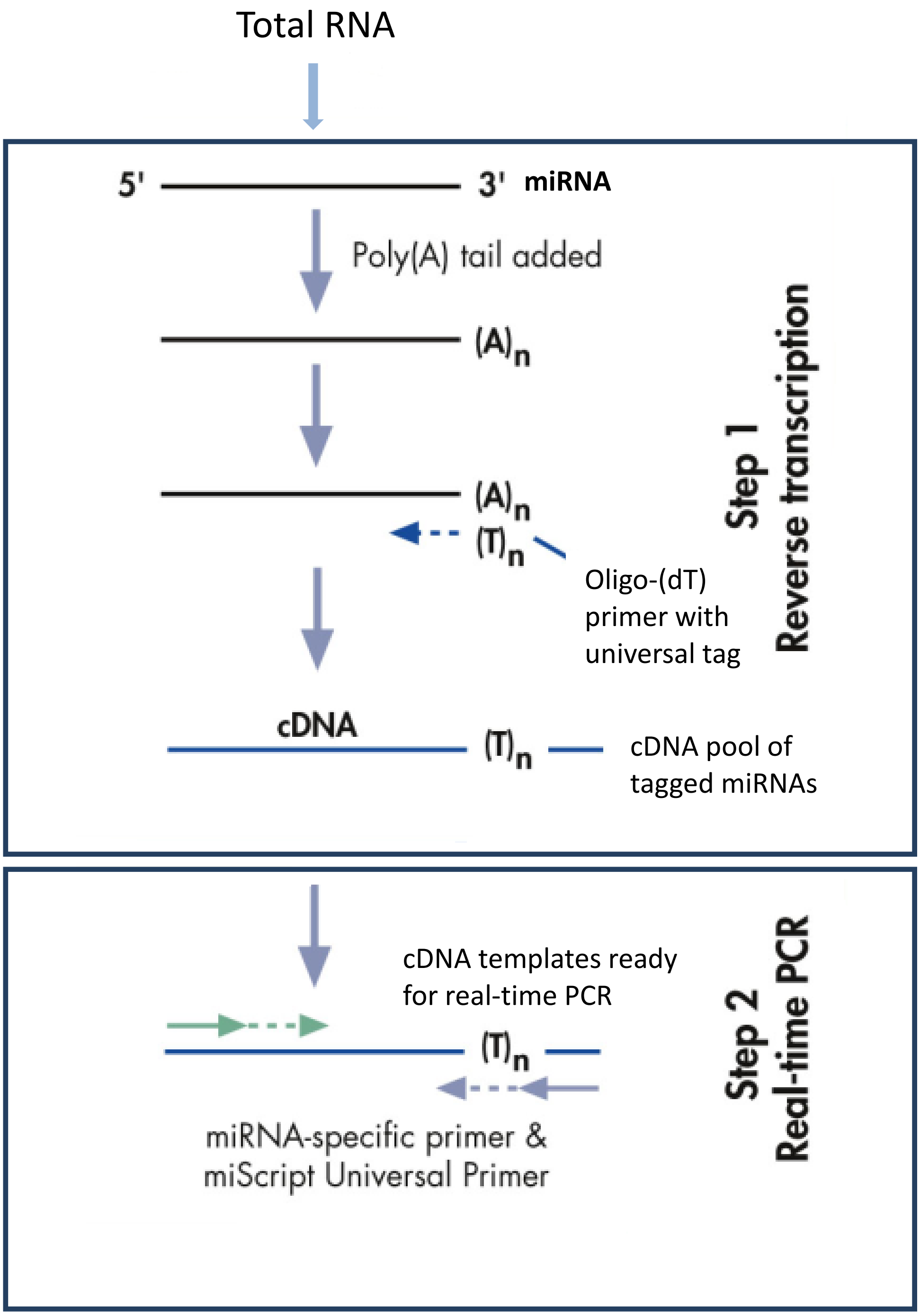


Figure 2-5. miScript principle. *Total RNA containing miRNAs, was polyadenylated by poly(A) polymerase and converted into cDNA by reverse transcriptase with oligo(dT)-primers. The cDNA was then used for real-time qPCR quantification of miRNA, using a miRNA-specific primer and a universal primer.*

Procedure:

1) 1 µl of undiluted total RNA samples were pipetted to a 96-well reaction plate.

2) Master mix components were mixed according the Kit (table 2-3) and 9 µl was added to each well on the reaction plate.

Table 2-3. Master mix for reverse transcription PCR for miRNA detection.
Modified from Qiagen miScript Reverse Transcription kit.

Components	Volume per well (ul)
miScript RT Buffer, 5x*	**4.0**
miScript Reverse Transcriptase Mix	**1.0**
RNase-free H_2O	**4.0**
Template RNA	**1.0**
Total per reaction	**10.0**

**Includes Mg^{2+}, dNTPs and primers*

3) The reaction plate was sealed with an optical adhesive cover and centrifuged at 1500 rounds per minute (rpm) for 1 minute to remove air bubbles and to spin down the content. The plate was placed on ice until loading the samples.
4) The cDNA synthesis was conducted using the thermal cycler (Eppendorf Mastercycler Gradient, Hamburg, DE), program shown in table 2-4.

Table 2-4. Thermal cycler program.

	Step 1	Step 2
Temperature	37° C	95° C
Time	60 minutes	5 minutes

5) Quantification of cDNA by NanoDrop™ 1000 Spectrophometer as described in chapter 2.4.1.1.
6) cDNA was stored at – 20° C until use.

2.4.3 Quantity and purity assessment of RNA and cDNA

RNA and cDNA quality includes both its purity; the absence of protein, DNA, carbohydrates, lipids and other compounds, and its integrity. In order to get accurate results, the RNA samples must be of the same quality when compared to each other. A partially degraded RNA sample would show some genes being lesser expressed compared to the expression profile of an intact RNA sample. Methods like real-time qPCR require high quality RNA, otherwise it could strongly affect the upcoming results. Even though it is advisable to check the RNA integrity, it could not be done in this study due to time limitations.

RNA and cDNA quantity and purity was measured using NanoDrop 1000 Spectrophotometer (Thermo Scientific, USA). The NanoDrop software measures any molecule`s absorbance in a sample. RNA and DNA absorbs at 260 nm, proteins and phenols absorbs light at 280 nm, and carbohydrates, salts and phenols absorbs at 230 nm. The 260/280 ratio is used to assess the purity of RNA and DNA, a ratio of 1.8 is accepted for pure DNA and a ratio of 2.0 is accepted for pure RNA. A lower ratio indicates the presence of phenols, proteins or other contaminants that absorb at or near 280 nm. The 260/230 ratio is another measure of purity, a ratio below 2.0 indicates contaminants that absorb at 230 nm (NanoDrop Technologies 2003).

Isolated RNA and cDNA from the reverse transcriptase PCR were assed for quantity and purity using NanoDrop ND-1000 software version 3.7.1. The elution solution from the RNA isolation kit was used as a blank measurement.

Measurement was performed according to manufacturer protocol:

1) The system was initiated with 1.5 µl distilled water sample.
2) “RNA-40” was chosen for RNA samples and “other-39” for cDNA samples.
3) A blank measurement was conducted using 1.5 µl RNase-free water.
4) 1.5 µl of the sample was pipetted onto the pedestal.
5) The system measured the absorbance of the sample giving the concentration in ng/µl.
6) The retention system was cleaned with a distilled water sample.

2.4.4 Real-time PCR

2.4.4.1 An improved method for quantifying gene expression

PCR is a powerful technique to detect and amplify fragments of DNA, and soon after its discovery in 1983 by Kari Mullis (Mullis *et al.*, 1986), this technique was used to quantify gene expression. Early quantitative PCR (qPCR) methods relied on end point analysis of PCR products, but this approach is both time consuming and not reliably quantitative because the lack of sensitivity. The more improved method, real-time qPCR, enables quantification of the PCR products in “real time”, during each PCR cycle. The PCR products are labelled and detected by using a fluorescently tagged substrate during the amplification process. This method is more time efficient and allows a precise quantification of the PCR products due to the high sensitivity of the fluorescent dye used for the detection of the amplification products. It also requires less RNA than end point assays, and is more resistant to nonspecific amplification (Fraga *et al.*, 2007; VanGuilder *et al.*, 2008).

2.4.4.2 Monitoring amplification

In this study the SYBR Green technology was used to label and detect PCR products. SYBR Green is an intercalating dye that fluorescence when binding to double-stranded DNA.
Since SYBR Green binds indiscriminately to double-stranded DNA it is important to perform a melting point analysis in order to identify the correct product. This is based upon the temperature-dependent denaturation of DNA, and is conducted by the real-time machine. By slowly increasing the temperature and monitoring the fluorescence, denaturation of different PCR products at different temperatures will be indicated by a sudden drop in fluorescence level. In this way, primer-dimers and PCR artifacts can easily be distinguished from longer and larger amplicon products. This is seen by the melting curve where fluorescence is plotted against temperature (figure 2-6). The peaks represent the points at which the maximum rate of change in fluorescence is detected, meaning where the different amplicons reached their melting point (Nolan *et al.*, 2006; VanGuilder *et al.*, 2008).

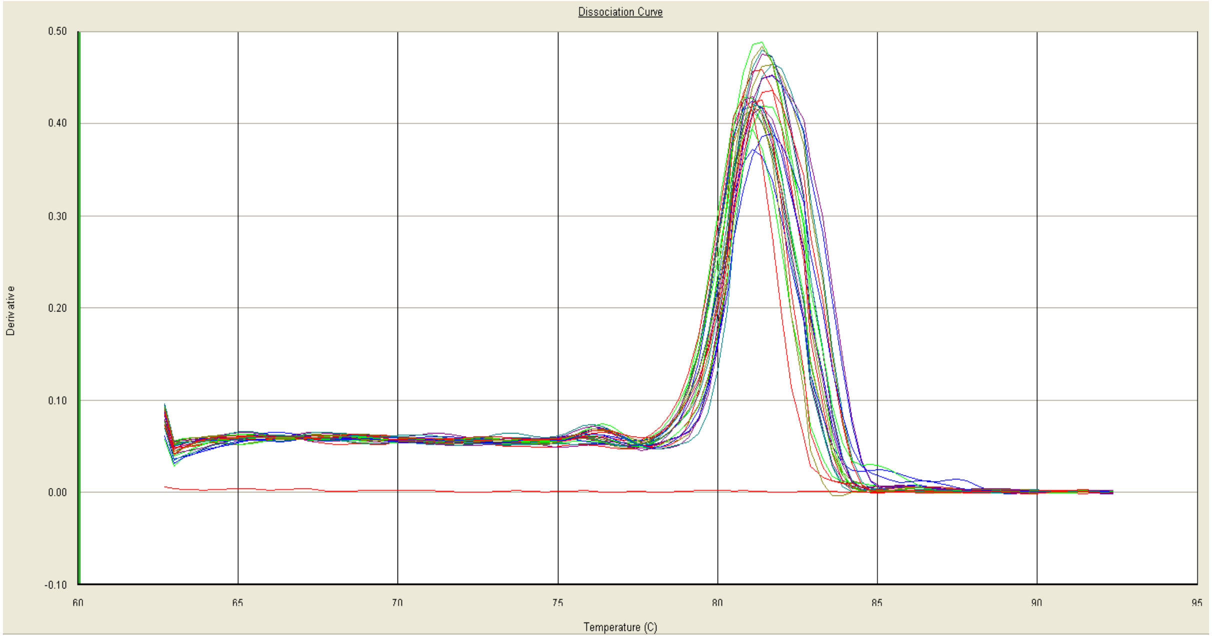


Figure 2-6 Melting point curve. *The rate of change is plotted against the temperature (x-axis), and a peak is shown at the melting point, illustrating when the amplicons separate.*

2.4.4.3 Primer design

Optimal primer design is critical for efficient amplification of target sequences. Several computer programs are available to assist in the design of primers, in this study Primer Express was used (primers are listed in appendix B). Several factors are taken into account when optimizing primer design; (1) Elimination of primer-dimers due to complementary sequence between primers, (2) annealing temperature among primers should match, and (3) suitable difference between the annealing temperature of the primers and the annealing temperature of the PCR product (Fraga *et al.*, 2007).

2.4.4.4 Real-time PCR set up

To identify possible changes of the selected mRNAs and miRNAs expression induced by BaP-exposure, 11 genes and 7 miRNAs were analyzed. These genes are listed in table 2-5 and 2-6.

Table 2-5. Target genes. *The selected genes were analyzed to identify expression changes induced by BaP-exposure.*

Gene symbol	Gene name	Gene function
Apex1	APEX nuclease (multifunctional DNA repair enzyme) 1	DNA repair
Neil1	Endonuclease VIII-like 1	DNA repair
Ogg1	8-oxoguanine DNA glycosylase	DNA repair
Xpc	Xeroderma pigmentosum, complementation group C	DNA repair
Xrcc1	X-ray repair cross-complementing protein 1	DNA repair
Cyp1a1	Cytochrome P450, family 1, subfamily A, polypeptide 1	Cytochrome P450 protein
Cyp1b1	Cytochrome P450, family 1, subfamily B, polypeptide 1	Cytochrome P450 protein
Dnmt1	DNA (cytosine-5-)-methyltransferase 1	Responsible for maintaining methylation patterns established.
Crem	cAMP responsive element modulator	Component of cAMP-mediated signal transduction during the spermatogenetic cycle.
Prm1	Protamine 1	Substitute for histones in the chromatin of sperm during the haploid phase of spermatogenesis.
Prm2	Protamine 2	

Table 2-6. Studied miRNAs.

Selected miRNAs analyzed to identify expression changes induced by BaP-exposure.

MiRNAs
mmu-mir-425
mmu-mir-34a
mmu-mir-191
mmu-mir-103
mmu-mir-26a
mmu-mir-16
mmu-let-7a

Real-time PCR for mRNA and miRNA detection was performed on Applied Biosystems 7500 Fast Real-Time PCR system, Absolute Quantification (Applied Biosystems). mRNA- and miRNA-specific primers were dissolved in TE-buffer to a concentration of 5 µM and aliquoted to the appropriate volume. Primers and master mix were kept at -20º C until use.

cDNA, master mix and primers were kept on ice when preparing for the real-time PCR run. The hoods were exposed to UV-light for 30 minutes before preparing the reactions.

2.4.4.4.1 Procedure for real-time PCR set up - mRNA:

A homemade SYBR Green master mix was prepared as shown in the appendix. A dilution series was carried out in order to determine the optimal cDNA dilution. From the dilution curve, a 1:10 cDNA dilution was selected as an optimal concentration and used in all qPCR runs. For each biological sample, three technical replicates were used. Standard curves for each target and reference gene were calculated from a 5 fold-serial dilution (1:1, 1:10, 1:100, 1:1000, 1:10 000) of cDNA from untreated sperm, testis and liver samples to find the optimal dilution during the real-time qPCR.

1) Preparing the reaction plate
   a. DNA hood: cDNA (diluted 1:10) and RNase-free water was added to each appropriate well of a standard optical 96-well plate, according to table 2-7.
   b. Master mix-hood: The PCR master mix with the enzyme Hot start Taq DNA polymerase was added in an 1.5 ml eppendorf tube. The appropriate amount of reverse and forward primer was added, and mixed by pipetting up and down.
   c. The plate was sealed with an optical adhesive cover and centrifuged at 1500 rpm for 1 minute at 4º C to spin down the content and to remove air bubbles.

Table 2-7. Reaction mix for real-time PCR, mRNA detection.

Components	Volume per well (µl)
Master mix*	10.0
Reverse primer	2.0
Forward primer	2.0
cDNA	2.0
RNase-free water	4.0
Total	**20.0**

*Contains Hot start Taq DNA polymerase

2) Programming the Real-time cycler

a. Assay: Absolute Quantification
b. Threshold value was manually adjusted to 0.02.
c. Detectors (gene name) and sample names were added to the appropriate wells.
d. Specifying thermal cycling conditions:
   i. Stage 1: 1 cycle
      (1) Step 1: 95 °C for 5 minutes
   ii. Stage 2: 40 cycles
      (1) Step 1: 94 °C for 30 seconds
      (2) Step 2: 60 °C for 1 minute
      (3) Step 3: 72 °C for 35 seconds (data collection step)
   iii. Stage 3: Dissociation stage was added.
   iv. Run mode was set as ‘Standard 7500’ and volume of each sample was 20 µl.

2.4.4.4.2 Procedure for real-time PCR set up - miRNA:

Real-time PCR for detection of miRNA was prepared using miScript SYBR Green PCR kit (Qiagen). No technical replicates were used, due to limited amount of master mix caused by late delivery of a new miScript kit.

Procedure:

1) Preparing the reaction plate
   a. The appropriate amount (table 2-8) of master mix, RNase-free water, universal primer and the miRNA-specific primer were mixed in an 1.5 ml eppendorf tube, and 9 µl added to each well in a 96-reaction plate.
   b. 1 µl of undiluted cDNA was added to each well along with the other reaction components.

Table 2-8. Reaction mix for real-time PCR, miRNA detection.

Components	**Volume per well (µl)**
2x QuantiTect SYBR Green PCR Master Mix	5.0
10x miScript Universal Primer	1.0
10x miScript miRNA-specific primer	1.0
RNase-free water	2.0
Template cDNA	1.0
Total volume	**10.0**

c. The plate was centrifuged as previously described in the procedure step 1.c, section 2.4.4.4.1

2) The real-time cycler was prepared as described in section 2.4.4.1, step 2.a-b.
   a. The thermal cycling conditions are shown in table 2-9.

Table 2-9. Cycling conditions for real-time PCR, miRNA detection.

Step	**Time**	**Temperature**
PCR Initial activation step	15 min	95º C
3-step cycling:		
Denaturation	15 s	94 º C
Annealing	30 s	55 º C
Extension	34 s	70 º C
Cycle number	35- 40 cycles	

2.4.4.5 Analysis of real-time PCR data

Quantification:

During the real-time PCR run the fluorescence is monitored providing an amplification curve. The amplification curve is a plot of the detected fluorescence versus the PCR cycles (figure 2-7). This curve is used for setting the "quantification point", further known as cycle threshold (Ct). The threshold determines the level of fluorescence signal that is sufficiently above background, which is considered to be a reliable signal. The cycle at which the threshold are met or exceeded are called the Ct value, and are used for quantification and for comparison between samples of the real-time PCR data (Duale 2010).

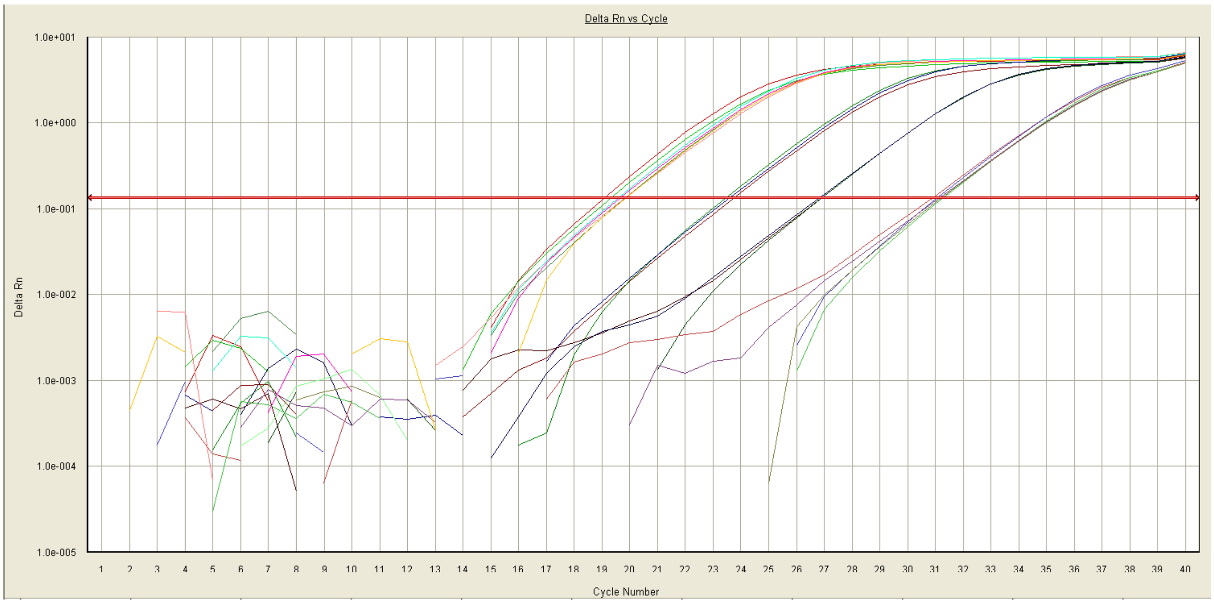


Figure 2-7. Amplification curve with the threshold line and Ct-values.

In this study, the expression level of mRNA and miRNA was measured using relative quantification. This method determines the changes in steady-state mRNA and miRNA levels of the target genes across multiple samples and expresses it relative to the levels of one or multiple reference gene(s) (Duale 2010). To calculate the expression level, the delta-delta Ct method (ΔΔCt) (Livak and Schmittgen 2001) was used. This method calculates the expression level by comparing the Ct values between the target genes and the reference genes (procedure for calculation of ΔΔCt, see appendix A). Optimal reference genes are equally expressed under experimental conditions and between all the samples to correct for methodological variations. In this study, 10 mRNAs and 3 miRNAs were evaluated as reference genes as described below (section: normalization).

For each PCR run, a non-template control (PCR master mix and water instead of template) was performed in order to rule out contamination. Melting point analysis was also conducted to identify possible primer-dimers and miss-priming.

PCR efficiency:

To reliably compare samples after a real-time PCR run, equal amplification efficiency is one important criterion (Duale 2010). The PCR efficiency can be estimated from the slope of a standard curve (figure 2-8): Efficiency = $10^{(-1/slope)}$-1. With 100 % efficiency, the amount of amplification is doubled. The PCR efficiency for the target and the reference genes should be between 90 % - 110 % (a slope between -3.1 and -3.6). In order to attain a high PCR efficiency, the PCR conditions should be optimized, which means determining the most favourable concentration of every component used in the PCR mix. This is time consuming, and within the time frame of this master thesis, PCR optimization could not be performed.

If the PCR efficiency differ more than 10 % between the target and reference genes, after optimizing the PCR conditions, the PCR data should be corrected by using mathematical calculations.

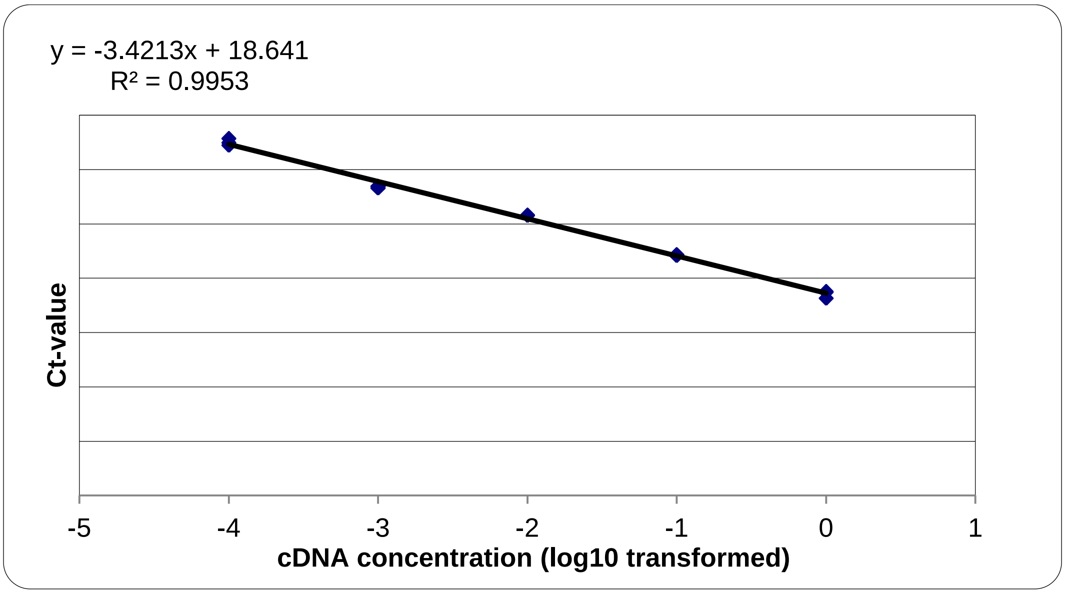


Figure 2-8. Standard curve. *A 5 fold-serial dilution of cDNA was used to calculate a standard curve from untreated liver to find the optimal dilution during the real-time PCR run. With a slope of -3.4 the PCR efficiency is close to 100 %. On the X-axis, the cDNA concentration is log(10) transformed, and the Y-axis shows the Ct-values.*

Normalization:

Due to the occurrence of experimental variations, such as differences in the amount and quality of starting material, as well as reaction efficiency, normalization are required to reduce such differences. The principle of normalization is to ensure identical cycling performance during real-time PCR, so that biologically relevant changes in mRNA levels are revealed (Vandesompele *et al.*, 2002). Up to date, there is no universally accepted method for normalization (Nolan *et al.*, 2006). In this study, a panel of 10 different reference genes were evaluated to normalize the target genes within sperm, testis, and liver. The use of several reference genes are thought to even out variations in the expression of these genes, and is seen as a robust approach for accurate normalization. Only the most stable expressed reference genes, selected by the BestKeeper algorithm software (Pfaffl *et al.*, 2004), were chosen to do the normalization. The software allowed for geometric averaging of the candidate reference genes, which then could be used to normalize for variations between runs in the real time PCR data. The reference gene expression from sperm, testis and liver samples are shown in the appendix A. Different reference genes normalized the target genes in sperm, testis and liver as shown in table 2-10.

The 7 selected miRNAs were normalized by 3 commonly used reference miRNAs; RNU43, U1SnRNA and U6 RNA. The expression of the reference mRNAs and miRNAs are shown in appendix A.

Table 2-10. Reference genes. *The most stable expressed reference genes were selected by the algorithm software BestKeeper. The X-marked squares illustrate which reference genes were used to normalize the different tissues; sperm, testis and liver.*

		Reference genes selected for each tissue		
Gene symbol	**Gene description**	**Liver**	**Testis**	**Sperm**
Tbp	Tata-box binding protein			
Pgk1	Phosphoglycerate kinase 1	X	X	X
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	X	X	X
18S rRNA	18S ribosomal RNA	X	X	X
Ywhaz	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta	X		
Gusb	Beta-glucuronidase	X	X	X
Actb	Beta-actin		X	
Rpl13a	Ribosomal protein L13a		X	
Tubb5	Tubulin-beta 5		X	X
Hprt1	Hypoxanthine phosphoribosyltransferase 1		X	

2.5 Statistical methods

Real-time PCR is considered as the gold standard for quantifying gene expression, yet a standard for statistical analyses of real-time PCR data are still lacking (Hellemans *et al.*, 2007). However, the analysis of variance (ANOVA) is a simple statistical method that can be used on real-time PCR data. ANOVA relies on assumptions of independence, equal variances and normality. The real-time PCR data are not always normally distributed nor possess equal variances. The real-time PCR data are not always normally distributed nor do they possess heterogeneity of variance across biological samples. This can be accounted for by applying a log 2 transformation. However, ANOVA is a robust test and can handle minor violation of the normality, as well as some differences in variances between the samples (Lovell and Omori 2008). We used one-way ANOVA to compare gene expression between treatments groups using the normalized Ct-value for each biological replicate; ΔCt BaP-exposed (target gene$_{Ct\text{-}value}$ – reference gene$_{Ct\text{-}value}$) versus ΔCt corn oil-exposed mice (target gene$_{Ct\text{-}value}$ – reference gene$_{Ct\text{-}value}$) versus ΔCt untreated mice (target gene$_{Ct\text{-}value}$ – reference gene$_{Ct\text{-}value}$). The data was tested for normality using Shapiro-Wilk test. If the equal variance test failed, the Tamhane's test was used. Dunnet's test was applied as post hoc test, to compare BaP-exposed mice to corn oil-exposed mice for assessing BaP-induced effects. The Kruskal-Wallis test, a distribution-free, non-parametric test was used if the normality and the equal variance test failed. Outliers were removed manually, by comparing the similarity of the three technical replicates.

Data regarding animal weight gain was compared using one-way ANOVA, followed by the post hoc Tukey's test. This test is similar to the Dunnet's test, but compares multiple samples, and is appropriate for unbalanced one-way ANOVA (unequal sample sizes). RNA yield and purity was analyzed using t-test or Mann-Whitney if the normality test failed. Some of the data were not tested for significance, such as the sperm count data subjected to hypotonic treatment, because chi-square test conducted on count data requires that each sample size is greater than or equal to 5, when our sample size was 2. The miRNA expression data had only two biological replicates and no technical replicates, making the power of the statistical analysis very small, and thus statistical analysis was not performed.

Sigmaplot version 11.0 was used to perform the statistical analyses and $p \leq 0.05$ was accepted as statistically significant. Sigmaplot do not have alternative tests for samples with unequal variances, therefore, SPSS version 17 was used to perform the Tamhane's test on the real-time PCR data.

3 Results

3.1 General toxicity of BaP

3.1.1 Effects of BaP on bodyweight

Exposure to BaP resulted in a significantly reduction in body weight gain. The BaP-exposed mice had a significantly lower body weight compared with the time-matched untreated mice after 24 days of exposure (table 3-1). Mice treated with BaP had a body weight gain of 2.5 %, followed by corn oil treated mice with 17.6 % weight gain and lastly the untreated mice with 20.7 % weight gain. Testis weight was compared relative to bodyweight, no significant differences were found among experimental groups ($p > 0.05$) (data not shown).

Table 3-1. Bodyweight gain in mice exposed to BaP or corn oil, and untreated mice.

Treatment	***Mean Bodyweight (g)***			**Mean bw gain (g)**
	n	Day 0	Day 24	Mean difference
BaP	10	23.5 ± 0.5	24.2 ± 0.8	0.6 ± 1.0*
Corn oil	6	22.1 ± 0.5	26.0 ± 0.6	3.9 ± 0.8
Untreated	4	23.7 ± 0.7	28.6 ± 0.8	4.9 ± 1.0

*Bodyweights are shown as mean ± SE. Day 0 was when BaP and corn oil were first injected, day 24 was the day of sacrifice. *Significant differences between BaP-treated mice and untreated controls (One-Way ANOVA, followed by post hoc Tukey test). No significant difference between BaP- and corn oil-treated mice.*

The observed *in vivo* BaP-induced reduction in body weight, shows that the exposure regiment has a certain toxicity. No other signs of general toxicity were observed. To investigate possible effects of BaP at molecular level, we conducted expression analysis of some selected genes and miRNAs. Before starting to examine gene expression changes some technical challenges must be dealt with, particularly, isolating high quality and sufficient amount of RNA from sperm cells. The results from the methodological modifications and improvements will be presented first.

3.2 Isolation of high-quality RNA

3.2.1 Isolation of pure sperm cells

One of the main goals of this study was to isolate pure sperm without contamination from somatic cells. To achieve this, the cauda epididymis was carefully trimmed to remove fat and blood vessels before harvesting of sperm cells. The crude sperm suspension was treated with a hypotonic buffer (SCLB) in order to lyse somatic cells present. Sperm cells and somatic cells were counted before and after hypotonic treatment to examine the effect of the lysis. Counting the sperm cells was only possible when the cells still were solved in the lysis solution. Removal of the lysis buffer made the cells stick together and they did not distribute evenly when diluted in water for cell counting. Therefore, the sperm cells were counted 20 minutes after the lysis solution was added, and at the same time the presence of any remaining somatic cells were counted (see table 3-2).

Table 3-2. Purity of sperm samples and effect of hypotonic treatment.

SCLB treatment	n	Sperm cells x 10^6	Somatic cells x 10^6	Proportion of somatic cells
Before	2	39.4 ± 0.9	0.4 ± 0.2	1.0 %
After 20 minutes	6	32.5 ± 1.9	0.07 ± 0.04	0.2 %

Cells are shown as mean ± SE values. The sperm cells counted were from untreated mice.

Before starting the hypotonic treatment, approximately 1 % of the sperm cell solution was somatic cells. 75 % of the present somatic cells were lysed during treatment. However, 20 % of the sperm cells were lost during the sperm purification procedure (data not shown).

3.2.2 Assessment of RNA yield and purity

3.2.2.1 Sperm RNA yield and purity assessment with/without addition of carrier RNA

Establishment of protocol for isolation of high-quality and sufficient amount of sperm RNA suitable for downstream analysis, such as real-time PCR, is another major goal of this study. We investigated whether addition of carrier RNA during sperm RNA isolation may improve the quality and yield of the extracted sperm RNA. Sperm samples with added carrier RNA had significantly higher total RNA yield and purity compared with sperm samples without carrier RNA added ($p<0.05$) (figure 3-1).

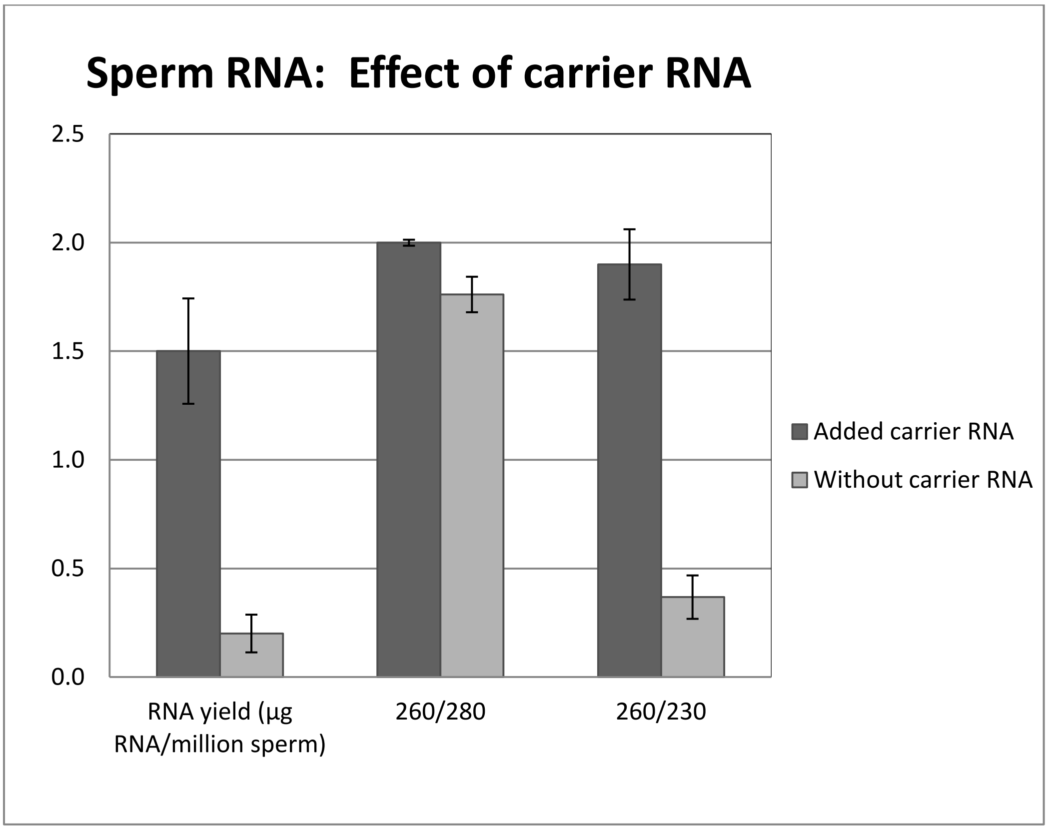


Figure 3-1. Comparison of total RNA yield and purity from two RNA isolation procedures. *Addition of carrier RNA (n = 20) and without (n = 6). The amount of added carrier RNA was subtracted from the final RNA yield. The 260/280 and 260/230 ratios measures RNA purity (see section 2.4.1.1). * Statistically significant difference (Mann-Whitney and t-test). RNA yield and purity is presented as mean ± SE.*

The addition of carrier RNA was found to greatly improve RNA yield and RNA purity. No significant difference in RNA quality was found between treatments (see appendix A). Based on these results, addition of carrier RNA was used in further downstream analysis.

3.2.2.2 RNA yield and purity assessment for testes and liver samples

To reliably compare gene expression between treatments, the quality of all isolated RNA samples should be approximately equal. Total RNA isolated from testis and liver samples are presented in table 3-3. Optimal RNA yield was obtained from both testis and liver samples, however, the 260/230 ratio obtained from liver samples was low, indicating the presence of some contamination from salts, carbohydrates and/or phenols. Furthermore, there were no significant differences in RNA yield and purity between the treatment groups (appendix A).

The calculated cDNA yield and purity for all samples are listed in appendix A.

Table 3-3. RNA yield and purity from liver and testis.

Tissue	n	Tissue (mg)	RNA (µg/mg tissue)	260/280	260/230
Testis	20	23.3 ± 2.1	2.4 ± 0.1	2.1 ± 0.01	1.8 ± 0.1
Liver	20	24.8 ± 1.3	3.4 ± 0.2	2.1 ± 0.01	1.4 ± 0.07

Amount of tissue used per RNA isolation, RNA yield and purity is shown as mean ± SE. No significant differences were found between BaP-, corn oil-treated and untreated mice.

3.3 Expression analysis of selected mRNAs and miRNAs

Investigation of mRNA and miRNA expression levels in male reproductive tissues, sperm and testis, might reveal possible sperm-quality markers and give mechanistic insight into reproductive effect of BaP.

To investigate BaP-induced effects on mRNA and miRNA expression, we compared the expression level in BaP-exposed mice to corn oil-exposed mice. The untreated controls were used to evaluate the effect of the corn oil alone.

Having established a procedure for isolation of sperm RNA, we wanted to examine whether we could reliably detect specific mRNAs and miRNAs in reproductive and somatic tissue.

3.3.1 Relative mRNA transcript levels in sperm, testis and liver following BaP-exposure

The relative mRNA transcript level were investigated for 11 selected genes in sperm, testis and liver samples from male mice exposed to BaP or corn oil and from untreated mice. The selected 11 genes take part in different cellular processes; DNA repair, BaP metabolism, sperm chromatin compaction and genes important for spermatogenesis. The relative transcript levels for these genes are presented in figure 3-2 to 3-4. Liver was used for comparison of somatic tissue to the reproductive tissue, sperm and testis.

3.3.1.1 Genes involved in DNA repair

Relative mRNA transcript levels for the five genes involved in DNA repair are presented in figure 3-2A - C. Testis and liver samples had similar expression profile for these genes compared to untreated controls, but the magnitude of the expression was different. Most of the genes in liver and testis were down-regulated following BaP- or corn oil-exposure (figure 3-2A & C). The net decrease in the expression level was more pronounced in the liver samples compared with testis samples. For the sperm samples, a slight up-regulation was observed for most of the DNA repair genes following BaP- or corn oil-exposure compared to untreated control samples (figure 3-2C). The marked effect of corn oil-exposure, especially on liver gene expression, was unexpected.

Significant effect of BaP-exposure was observed in sperm, *apex1*, and in testis, *xrcc1*, by comparing BaP-exposed mice to corn oil-treated mice. No effect of BaP-exposure was observed in liver. There was also a significant effect of corn oil-exposure *xpc* gene in testis, by comparing corn oil-treated mice to untreated mice.

Constitutive expression:

The constitutive expression pattern for the five DNA repair genes was similar between sperm and liver (figure 3-2 D). The expression level for testis samples was slightly higher than the other two tissues, in particular of *xrcc1*.

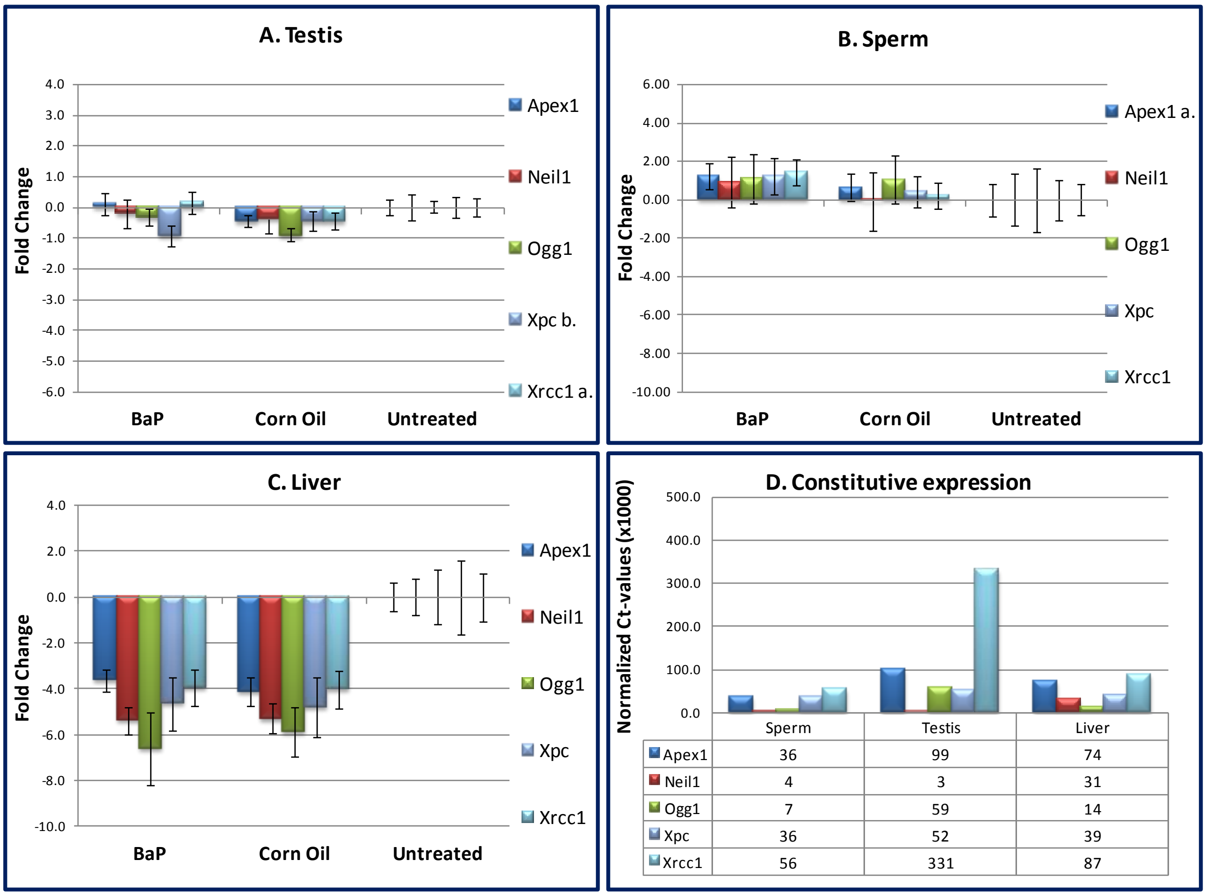


Figure 3-2. A-D. Expression of the DNA repair genes; *apex1*, *neil1*, *ogg1*, *xpc* and *xrcc1*. *A. - C.* *The relative mRNA expression of BaP- and corn oil- treated mice compared to untreated controls. Data is presented as log2 transformed 2 $^{-\Delta\Delta Ct}$ values. A. The bars represent the average fold change with error bars representing the standard error of the mean. The treatments are indicated below. a. Indicates significant effect of BaP-exposure; BaP-exposed mice compared to corn oil-exposed mice. b. Indicates significant effect of corn oil-exposure; corn oil-exposed mice compared to untreated mice.* ***D.*** *Gene expression data from untreated mice. Data is presented as the normalized Ct-value (2 $^{-\Delta Ct}$) and multiplied by a factor of 1000. The table below depicts the values of the mRNA expression data.*

3.3.1.2 Genes related to sperm nuclear condensation

The relative transcriptional response of the genes related to nuclear condensation in sperm, *prm1* and *prm2*, are shown in figure 3-3 A – C. Testis and liver samples showed a reduced expression compared to untreated control, but liver had a more pronounced decrease compared to testis. In sperm, *prm1* had decreased levels of expression compared to the untreated control, as oppose to *prm2*.

Increasing protamine 1: protamine 2 (prm1:prm2) ratio at protein level in sperm, have shown to be associated with infertility in human (Oliva2006). In our results, an alteration of *prm1*:*prm2* ratio at the mRNA expression level between treatments in testicular cells was observed (figure 3-3 B & D). The changes in *prm1*:*prm2* ratio between treatments is illustrated in table 3-4, showing increasing ratios from untreated to treated mice (BaP- and corn oil-treated). This difference did not reach significance. *Prm1* had a 27 % decrease in gene expression from untreated to BaP-treated mice, while *prm2* showed a 44 % decrease.

Table 3-4. Alteration of prm1: prm2 ratio between treatments in testis.

Treatment	*prm1*	*prm2*	*prm1:prm2*
Bap	43.4 ± 0.2	7.3 ± 0.3	6.0 ± 0.4
Corn oil	38.7 ± 0.2	6.5 ± 0.2	5.9 ± 0.2
Untreated	59.4 ± 0.3	13.0 ± 0.3	4.6 ± 0.4

The protamine expression level is presented as mean of $2^{-\Delta Ct}$ *values ± SE. No significant difference was found between the ratios (one-way ANOVA).*

In contrast, in sperm a tendency of a reduced *prm1*:*prm2* ratio was observed (table 3-5).

Table 3-5. Alteration of *prm1: prm2* ratio between treatments in sperm.

Treatment	*prm1*	*prm 2*	*prm1:prm2*
BaP	0.03 ± 0.8	0.4 ± 0.4	0.1 ± 0.9
Corn oil	0.04 ± 0.7	0.5 ± 0.4	0.1 ± 0.8
Untreated	0.10 ± 1.2	0.2 ± 0.6	0.5 ± 1.4

The protamine expression level is presented as mean of $2^{-\Delta Ct}$ *values ± SE. No significant difference was found between the ratios (one-way ANOVA).*

Constitutive expression:

The constitutive expression in the testes was very high, compared to the sperm and liver samples, reflecting the importance of protamines for chromatin condensation during sperm maturation.

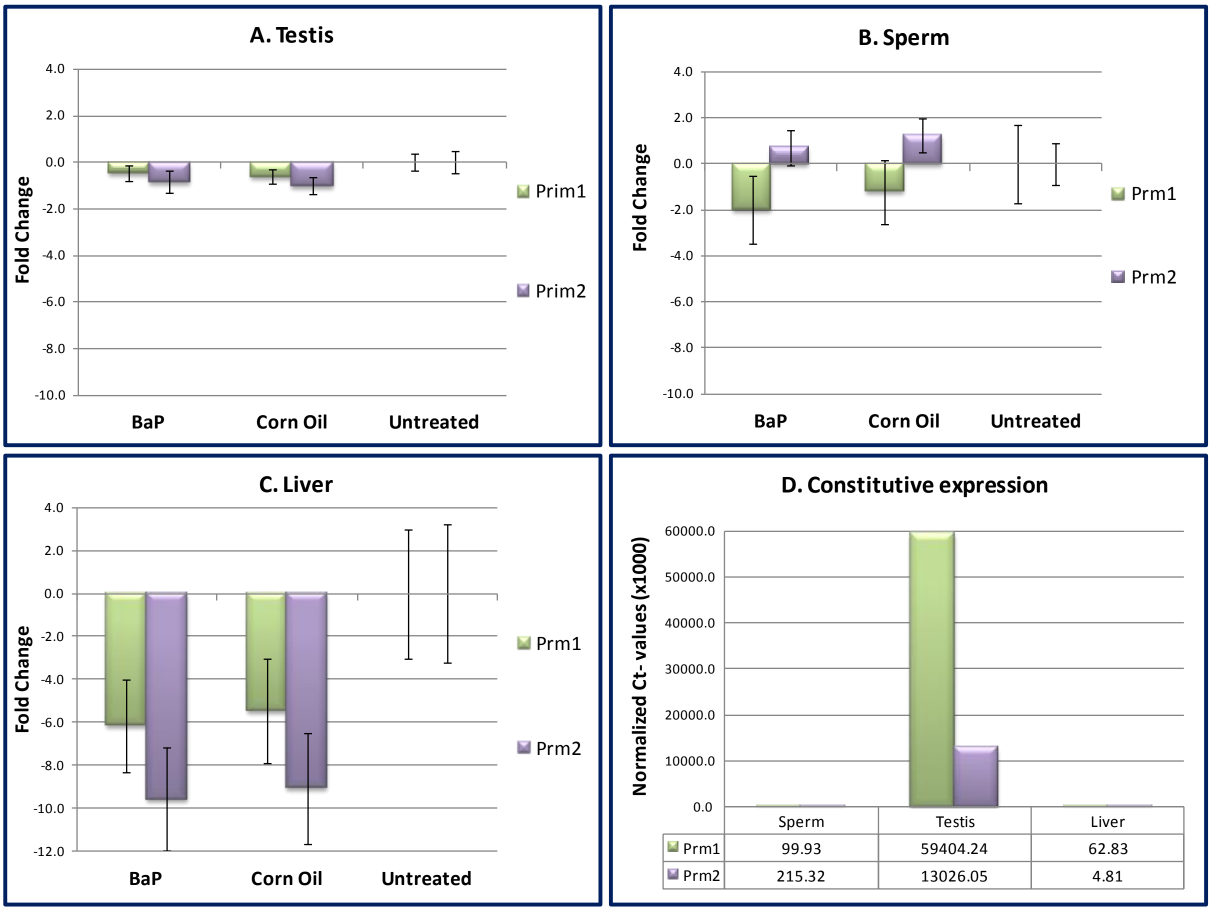


Figure 3-3. A-D. Expression of protamine genes; *prm1 and prm2.* A. - C. *The relative mRNA expression of BaP- and corn oil- treated mice compared to untreated controls. Data is presented as log2 transformed 2* $^{-\Delta\Delta Ct}$ *values. The bars represent the average fold change with error bars representing the standard error of the mean. The treatments are indicated below. No significant differences were found between treatments.* ***D.*** *Gene expression data from untreated mice. Data is presented as the normalized Ct-value (2* $^{-\Delta Ct}$*). The table below depicts the values of the mRNA expression data.*

3.3.1.3 Genes related to BaP metabolism and spermatogenesis

Liver and testis showed similar expression between the BaP- and corn oil-treated mice, but liver had a larger down-regulation (figure 3-4A & C). In sperm samples, a slight induction was observed in BaP-exposed mice compared to corn oil-exposed mice (figure 3-4B).

No significant effect of BaP was observed in liver or in testis. In sperm, *cyp1b1* showed a significant effect of BaP-exposure (figure 3-4B).

Constitutive expression:

The expression of *cyp1a1* was low in all tissues, reflecting the low constitutive expression of this highly inducible gene. For the three remaining three genes, the constitutive expression was higher in testis, compared with liver and sperm samples (figure 3-4D). Testis showed high expression of *Crem*, an enzyme involved in spermtaogenesis (Miller2011). *Cyp1b1*, which is known to be induced by BaP in liver, was constitutively expressed lower in liver than in testis.

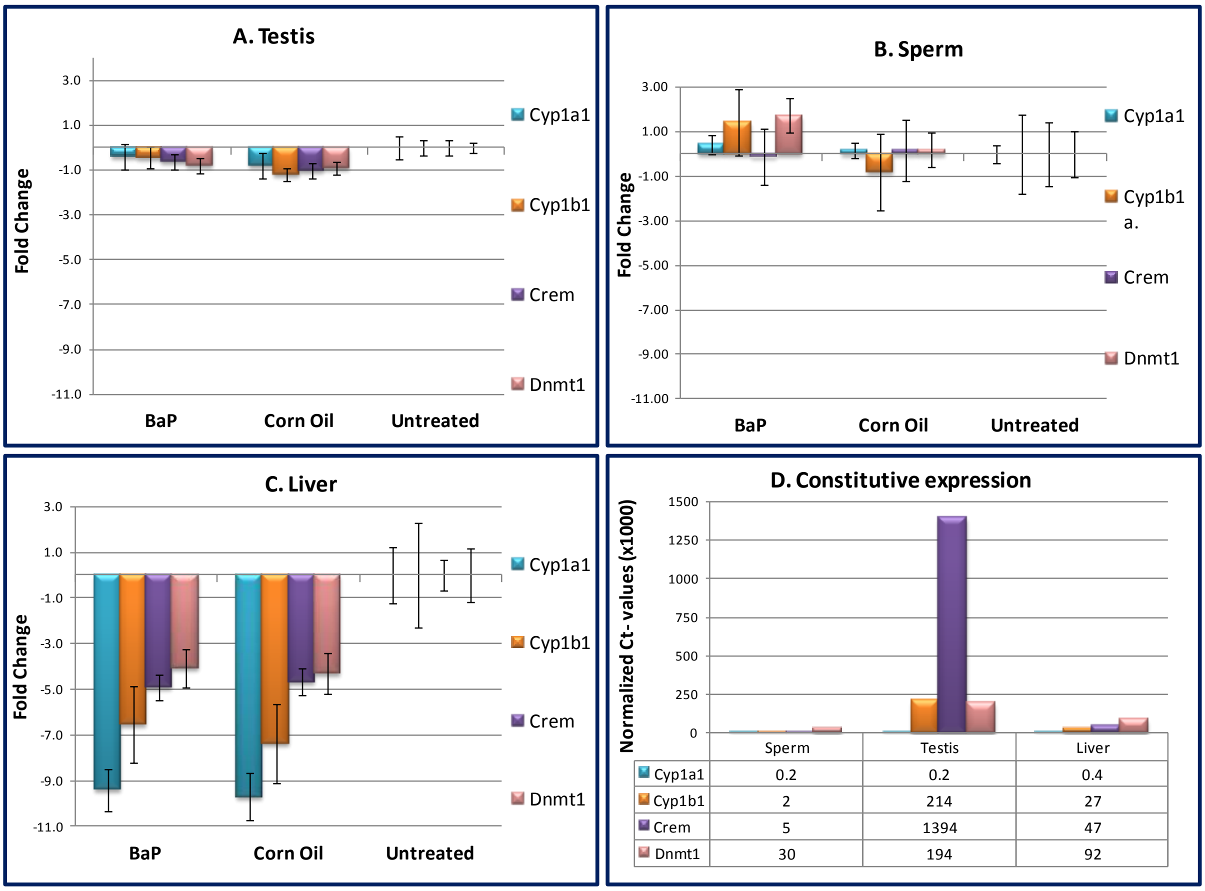
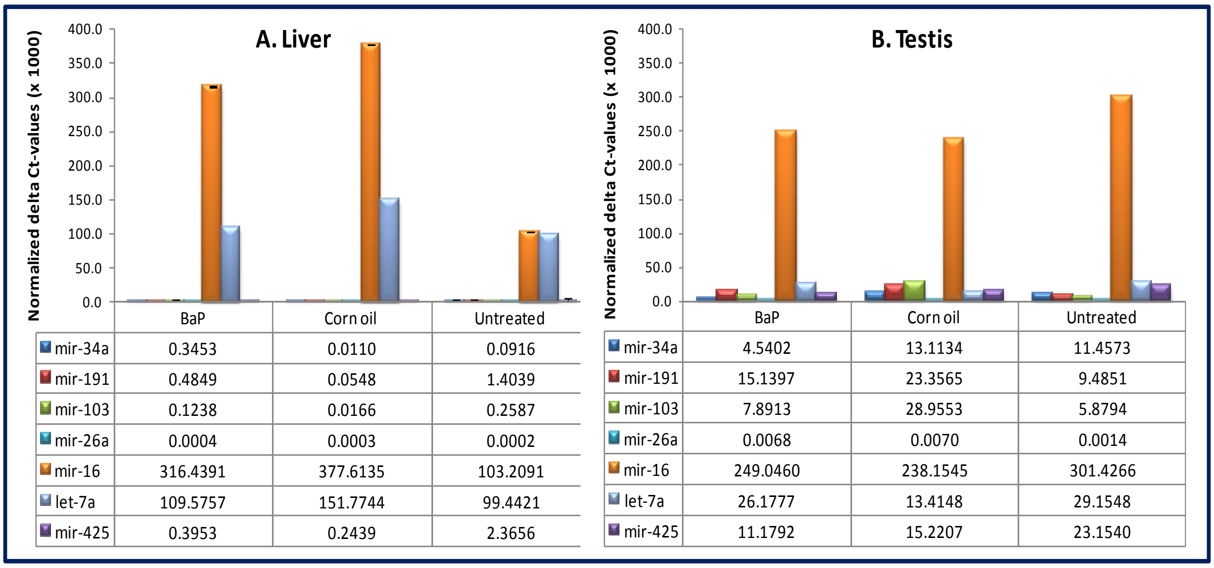


Figure 3-4. A-D. Expression of BaP metabolism genes; *cyp1a1*, *cyp1b1* and spermatogenesis genes; *crem* and *dnmt1*. *A. - C*. *The relative mRNA expression of BaP- and corn oil- treated mice compared to untreated controls. Data is presented as log2 transformed 2* $^{-\Delta\Delta Ct}$ *values. The bars represent the average fold change with error bars representing the standard error of the mean. The treatments are indicated below. a. Indicates significant effect of BaP-exposure; BaP-exposed mice compared to corn oil-exposed mice.* ***D****. Gene expression from untreated mice, presented as the normalized Ct-value (2* $^{-\Delta Ct}$*). The table below depicts the values of the mRNA expression data.*

3.3.2 MiRNA expression analysis

Changes in miRNA expression level were examined for 7 miRNAs in sperm, testis and liver from mice exposed to BaP or corn oil, compared to untreated mice (figure 3-5 to 3-6). The miRNA expression data is presented as normalized Ct-values ($2^{-\Delta Ct}$).

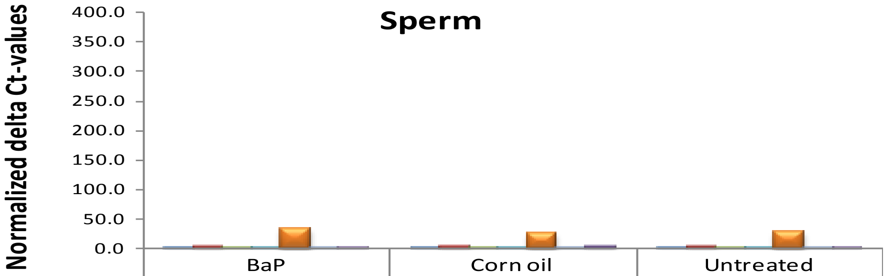
Because no technical replicates were used, and there were only two biological replicates per treatment group it is difficult to evaluate the effect of treatment on miRNA expression and statistical tests were not performed. However, several of the miRNAs were readily detectable also in sperm samples. The miRNAs showed no clear differences in expression patterns between BaP-, corn oil- and untreated controls, except for a lower expression of mir-16 in liver of untreated mice. The expression of mir-16 was considerably higher than the other miRNAs examined in all the three tissues, with similar expression levels in liver compared to testis. In liver, Let-7a was also highly expressed, whereas the remaining miRNAs showed low expression. Except from mir-26a that was hardly detectable in any of the tissues examined, all miRNAs showed a significant expression in testis. In sperm, mir-191 was relatively highly expressed in addition to mir-16.



A. Liver	BaP	Corn oil	Untreated
mir-34a	0.3453	0.0110	0.0916
mir-191	0.4849	0.0548	1.4039
mir-103	0.1238	0.0166	0.2587
mir-26a	0.0004	0.0003	0.0002
mir-16	316.4391	377.6135	103.2091
let-7a	109.5757	151.7744	99.4421
mir-425	0.3953	0.2439	2.3656

B. Testis	BaP	Corn oil	Untreated
mir-34a	4.5402	13.1134	11.4573
mir-191	15.1397	23.3565	9.4851
mir-103	7.8913	28.9553	5.8794
mir-26a	0.0068	0.0070	0.0014
mir-16	249.0460	238.1545	301.4266
let-7a	26.1777	13.4148	29.1548
mir-425	11.1792	15.2207	23.1540

Figure 3-5. A & B. MiRNA expression in liver and testis. *The miRNA expression is presented as normalized Ct-values ($2^{-\Delta Ct}$), the table depicts the expression values. The treatments are indicated below the x-axis.* ***A**. – **B.** Liver showed high expression of let-7a, likewise for mir-16, which also was high expressed in testis.*



	BaP	Corn oil	Untreated
mir-34a	0.2258	0.1791	0.0865
mir-191	4.3333	5.1070	3.7264
mir-103	0.2067	1.3126	0.7109
mir-26a	0.0010	0.0020	0.0008
mir-16	33.7184	26.7687	28.7961
let-7a	0.8267	1.2163	1.7027
mir-425	1.0739	3.6900	1.1443

Figure 3-6. MiRNA expression in sperm. *The miRNA expression level data is presented as normalized Ct-values ($2^{-\Delta Ct}$), the table below depicts the values of the expression. The treatments are indicated below.*

4 Discussion

The sperm-quality in the western world is low and a decline during the past decades has been suggested (Andersson *et al.*, 2008). In addition, there are reports indicating a relationship between sperm-quality and increased disease susceptibility and mutations in the offspring (Ahmadi and Ng1999; Macklon *et al.*, 2002). The actual reasons for this are not yet established, but the genetic integrity of the sperm is clearly important for male fertility and the health of the offspring. Environmental exposure is thought to be associated with the proposed decline in male fertility, for this reason, identification of sperm-quality marker would help to understand the causes of male infertility and to identify male patients in the clinic. To date, a large part of male infertility cases remains unexplained, and robust methods to assess fertility are clearly needed.

In search for a novel sperm quality marker, the suitability of using sperm RNA was investigated in this study. The expression changes in some selected mRNAs and miRNAs in sperm was investigated following BaP-exposure. The BaP-induced expression changes in testis and liver were also examined as comparison to sperm samples. In order to investigate mRNA and miRNA expressions in sperm, a protocol for isolating pure sperm samples and good quality RNA from sperm first had to be established.

The dose of BaP used in this study (3 x 50 mg/kg bw) is much higher than what human normally would be exposed to. The BaP dose used in this study has been reported to cause DNA damage in testis, sperm and liver (Olsen *et al.*, 2010; Verhofstad *et al.*, 2010a), as well as changes in gene expression *in vitro* (Akerman *et al.*, 2004; Hockley *et al.*, 2007; Yu *et al.*, 2000) and in vivo (Baken *et al.*, 2008; Bartosiewicz *et al.*, 2001). Changes in miRNAs after BaP-exposure have also been studied, but minor changes were observed (Malik *et al.*, 2012; Yauk *et al.*, 2011). However, these reported changes in gene expression were observed from 6 hours to a few days following BaP-exposure, whereas we analyzed gene expression changes after 24 days. This time point was chosen in order to expose the developing germ cells at a stage where they are transcriptionally active. The high dose was used to increase the possibility of observing BaP effects at gene expression level. In addition, the effect of genotoxic chemicals is assumed to be linear with the dose, and a high dose of BaP can therefore be used to extrapolate low dose effects.

In the present study, we showed that it is possible to obtain good quality RNA in sufficient amount for mRNA and miRNA analysis from a single mouse. Previous studies isolating sperm RNA used pooled samples from several individuals, increasing the amount of RNA which makes it easier to extract. Furthermore, we observed some changes in mRNA and miRNA expression of the selected genes, in response to administration of BaP. To our surprise, we also found expression changes in response to corn oil-exposure.

4.1 Gene expression methodological considerations

4.1.1 Purity of isolated sperm and evaluation of RNA quality

Somatic cells contain ~600-fold more RNA than sperm cells (Galeraud-Denis *et al.*, 2007). Therefore, obtaining pure sperm RNA without contamination from somatic cells was critical for correct quantification of sperm transcripts. Careful dissection of the cauda epididymis to remove fat and blood vessel as well as hypotonic treatment was used to remove such contamination. Hypotonic treatment is often used for analysis of human sperm. The hypotonic treatment was used to remove such contamination, as its efficacy is known from previous studies (Goodrich *et al.*, 2007; Ostermeier *et al.*, 2002). The hypotonic treatment lysed most of the somatic cells in the sperm samples, from being 1 % before treatment to 0.2 % after treatment. The somatic cells were counted after 20 minutes of lysis, and then centrifuged, allowing aspiration of the lysis solution from the sperm pellet. Some of the sperm samples were also checked for the presence of somatic cells after the centrifugation step, at this point; no somatic cells were observed. Therefore, the amount of somatic cells present after hypotonic treatment might be even lower than 0.2 %. This indicates that mechanical stress might be necessary for a complete removal of somatic cells. The reason why cell count was performed prior to centrifugation, was due to the excessive loss of cells when aspirating the lysis solution from the sperm pellet after centrifugation, and because the cells clumped together made it difficult to perform a correct count. For this reason, the contribution of somatic cells might have been smaller than our data showed. To have a measure of somatic cell contamination, the use of primers specific for somatic cells would be useful.

The use of high quality RNA is critical for obtaining meaningful gene expression data. In somatic cells, the ratio between 28S and 18S rRNAs is often used as an indication of the quality of the total RNA, and is conducted using e.g. a BioAnalyzer 2100 to determine the integrity number (RIN) (Fleige and Pfaffl 2006). The RIN value is dependent on the presence of rRNA, but because it is absent in sperm (Betlach and Erickson 1976), such measure of quality cannot be used. Instead of BioAnalyzer, Nanodrop was used as a measure of RNA quality. While the RIN number reflect unspecific damage to the RNA, such as sample mishandling and postmortem degradation, the Nanodrop only indicates presence of contaminants which can act as PCR inhibitors. Another method that may be useful for assessing the integrity of sperm RNA, is the use of sperm-specific primers (Lalancette *et al.*, 2009). This is to demonstrate that the sperm RNA carry transcripts of full-length and are not degraded (Ostermeier *et al.*, 2005a). However, we did not find such primers.

4.1.2 Addition of Carrier RNA increased the sperm RNA yield

Successful use of carrier RNA to enhance recovery of DNA and miRNA from dilute solutions has been reported by several studies (Andreasen *et al.*, 2010; Gallagher *et al.*, 1987). In this study, the addition of carrier RNA improved the extraction efficiency of sperm RNA, and provided high-yield and quality RNA. However, measuring the sperm RNA quality using Nanodrop, the signal from the carrier probably dominated over the sperm RNA, but it is still useful for assessing the presence of contaminants which may cause PCR inhibition. The addition of an RNA carrier improves the yield of RNA in challenging samples such as sperm.

Although the carrier RNA was present during the quantification procedure (real-time PCR), the use of mouse specific primers made it unlikely that the carrier, yeast tRNA, would bind the primers. However, the use of carrier RNA as a template would help to reveal any unspecific amplification. In addition, sperm specific primers would have been a reliable method to ensure correct quantification of the sperm RNA as long as they are not altered by treatment. *Prm1* and *prm2* were first assumed to be sperm specific, but surprisingly they showed constitutive expression, although low, also in liver. Whether *prm1* and *prm2* are present in the contaminating leukocytes are not known. Further literature search revealed findings on protamine genes being present in liver tissue (Nemoto *et al.*, 2011).

4.2 Corn oil-induced changes in mRNA and miRNA expression

The most unexpected finding from this study, was that corn oil-treated mice showed similar mRNA expression pattern as those treated with BaP in liver, testis and sperm. This was also observed in the examined miRNAs. Fatty acids have been reported to induce *cyp1a1* in human cells involving another pathway than the Ah-receptor (Villard *et al.*, 2011). Another study observed a decrease in mRNA expression of cyp-enzymes (*cyp2a4/5*) after sub-chronic exposure (Kojo *et al.*, 1996). This may suggest that components in corn oil have the ability to cause changes in genes expression (other genes than lipid metabolism-related). It has been suggested that corn oil are susceptible to peroxidation and production of free radicals, and mice fed with corn oil-enriched diet showed increased oxidative damage in liver because of a decrease in the antioxidant defense system (Domitrovic *et al.*, 2006). Mice exposed to acrylamide (ACR) and fed with a corn oil and pork fat diet, potentiated the ACR-induced oxidative stress in epididymal sperm. This may indicate that mice exposed to corn oil in our study were subjected to oxidative stress, and may have an effect on the observed induction of mRNA and miRNA expression (Zhang *et al.*, 2010).

The fairly large amounts of corn oil used as solvent for BaP and as vehicle control should be considered when studying gene expression profiles in mice. The total volume of the injected corn oil was ~450 µl, and this may be too high, when considering the bodyweight of a mice (~24 g), although being in accordance with current recommendations of maximum 2 ml/kg bw. Furthermore, the quality of the oil used may play a role.

In testis, the *xpc* gene was significantly induced by corn oil. *Xpc* takes part in the repair of bulky DNA adducts formed by reactive metabolites of BaP, and have been reported to be differentially regulated after BaP-exposure (Verhofstad *et al.*, 2010b). However, this effect was observed 4 days following BaP-exposure, indicating that the effect of BaP on *xpc* expression decreased after this timepoint.

4.3 BaP-induced changes in mRNA and miRNA expression

In an attempt to identify sperm-quality markers, BaP-induced changes of some selected mRNAs and miRNAs were analyzed in sperm, testis and liver. In our study, we hoped to detect a persistent change in mRNA and miRNA expression in sperm and possibly also in testis.

4.3.1 mRNA transcriptional response following BaP-exposure

Some BaP-induced changes in mRNA expression compared to corn oil controls, were observed in sperm and testis, with the largest gene expression modulation was observed for dnmt1 gene in sperm ~ 3.33 (not significant). In liver, none of the genes investigated were induced by BaP. Due to the late timepoint of gene expression examination, we did not expect to observe any effects of BaP in liver tissue, as the gene expression level goes back to normal within days of BaP-exposure. This was confirmed by the lack of induction of the cyp-genes, *cyp1a1* and *cyp1b1*, which are highly inducible by BaP metabolites. Meier (2008) reported a high induction of *cyp1a1* and *cyp1b1* by BaP after 1 day (~4000 fold) of exposure in liver, but then it rapidly decreased to near baseline at day 17 after exposure. This suggests that *cyp1a1* and *cyp1b1* expression may further declined below baseline at day 24 after BaP-exposure, which is what our results indicated. A similar trend was observed for *cyp1a1* in testis (Meier 2008).

Two DNA repair genes, *apex1* in sperm, and *xrcc1* in testis, and one BaP-metabolizing gene, *cyp1b1* in sperm, were significantly induced by BaP compared to corn oil controls. However, the standard errors of these genes in sperm samples were fairly large, and when considering the modest change in gene expression for all the three genes (~2 fold-change in *cyp1b1* and *apex1*), it becomes difficult to distinguish biological relevant changes from changes caused by natural background variations. Technical variations must therefore be kept at a minimum level in order to detect biological relevant changes. Possible causes of these sources of variability, are discussed in section 4.4.

Overall, the BaP effect on the selected mRNA was modest, indicating little persistent change of the selected genes after BaP-exposure. However, some potentially interesting trends were observed for the protamine genes, *prm1* and *prm2.*

4.3.1.1 Altered protamine expression – Possible indicator of sperm-quality

The main objective for this master thesis, was to examine the potential of gene expression changes in sperm and possibly testis as an indicator of sperm-quality. Sperm from infertile men has been shown to have altered prm1:prm2 ratio at protein level (Oliva2006), compared to fertile men (Aoki *et al.*, 2006; Carrell and Liu 2001; De *et al.*, 1998). The expression level of the protamine genes; *prm1* and *prm2*, have also been examined in both testicular cells and sperm, and their transcript levels have been shown to vary between fertile and infertile men (Lambard *et al.*, 2004; Steger *et al.*, 2008).

In this study, we investigated changes in the *prm1:prm2* ratio at gene expression level, and a slight increase in the *prm1:prm2* ratio from untreated to BaP- and corn oil treated mice was detected in testis; 4.6 ± 0.4 in untreated mice, 6.0 ± 0.4 in BaP-exposed and 5.9 ± 0.2 in corn oil-exposed mice (not significant). The amount of both *prm1* and *prm2* decreases after treatment (BaP or corn oil), but *prm2* show a larger reduction with a 44 % decrease compared to 27 % decrease of *prm1*. A larger change in *prm2* compared to *prm1* in testis of infertile men was demonstrated in a study comparing fertile and infertile men (Steger *et al.*, 2008), suggesting different mRNA stability for the two genes, with *prm2* being more susceptible to variation than *prm1* (Lewis *et al.*, 2003). In sperm cells, a marked decrease of the *prm1:prm2 ratio* was observed between the different treatments; 0.5 ± 1.4 in untreated mice, 0.1 ± 0.9 in BaP-exposed mice and 0.1 ± 0.8 in corn oil-exposed mice. However the differences between untreated and treated samples were not significant. Interestingly, a similar decrease in *prm1:prm2* ratio was observed in ejaculates of infertile men compared to fertile men, 1:1.17 and 1:1, respectively (Steger *et al.*, 2008). Thus it appears that infertility in men may be associated with an increased *prm1:prm2* gene ratio in testis and a reduced ratio in sperm.

Most studies have investigated the protamine ratio at the protein level, and there are evidence that links high DNA fragmentation indexes, to reduced sperm DNA integrity (Aoki *et al.*, 2005), decreased protamine content and lower *in vitro* fertilization (IVF) and intra cytoplasmic sperm injection (ICSI) rates. At the mRNA level, the *prm1:prm2* ratio in round spermatids of infertile men (testicular cells), have also been shown to be related to successful IVF (Steger 2001). In this study, the BaP- and corn oil-induced alteration of *prm1:prm2* ratio in both testis and sperm, including the reduction of both transcripts in testis, might indicate that corn oil have an effect on mRNA protamine expression although it was not significant. Possible mechanism that may be responsible of the dysregulation of *prm1* and *prm2* mRNA expression, have been explained by an abnormal functioning of a regulator of transcription, translation or post-translation modification that affect not only protamines, but also genes involved in spermatogenesis (Steger *et al.*, 2008). It is possible that corn oil is capable to disturb some of these processes, possibly by oxidative damage, which may lead to altered transcription and translation of the protamines, causing damage to the sperm DNA.

The *prm1* and *prm2* transcripts may be suitable as predictive markers for sperm-quality, however, the changes in prm1 and prm2 were only modest and further research must be done.

4.3.2 Constitutive expression of the examined mRNAs

The high constitutive expression of DNA repair genes involved in the Base Excision Repair pathway (BER); *apex1, ogg1* and *xrcc1* in testis, is consistent with previous studies (Ji *et al.*, 2010). They indicated that genes related to the BER pathway played a role in the maintenance of genetic integrity during spermatogenesis, therefore high expression of BER pathway genes are observed in rodents and primates testis (Walter *et al.*, 1994).

Both testis and liver had low constitutive expression of *cyp1a1,* whereas testis had a somewhat higher expression of *cyp1b1,* which is in accordance with previous reports (Shimada *et al.*, 2003). In sperm, the constitutive expression of *cyp1a1* and *cyp1b1*, was very low.

The constitutive expression of the spermatogenesis related genes; *crem* and *dnmt1* was higher in testis than in liver and sperm. The high expression of *crem* in testis, an important gene during spermatogenesis, agrees with previous studies (Beissbarth et al., 2003). The product of the *Dnmt1*gene is responsible for maintenance of methylated DNA in replicating cells, and is reported to be constitutively expressed in testicular cells which is in accordance with our findings (Omisanjo *et al.*, 2007).

The protamines are highest constitutively expressed in testis (Oliva2006) as consistent with our results. In testis, the genes are transcribed and stored in spermatocytes and round spermatids for later translation in elongating spermatids (Oliva2006). Expression of *prm1* and *prm2* genes were also detected in liver, which have been reported in previous studies as well (Nemoto *et al.*, 2011).

4.3.3 MiRNA expression

Sperm miRNA has been hypothesized to play a role in early embryonic development and may be potential markers of exposure and/or effect. One important aim was to extract and detect miRNA from individual sperm samples. MiRNAs were successfully detected in sperm samples, and also in testis and liver samples. The expression pattern within each tissue and between the three treatment groups, was similar, except for the somewhat lower expressed mir-16 in liver of untreated mice.

Due to limited amount of the components needed to run real-time PCR for detection of miRNA, only two biologically samples were used per treatment group and no technical replicates. Hence, care has to be taken when interpreting our miRNA results.

The miRNAs; mir-16 and let-7a, were highly expressed in liver of BaP- and corn oil-exposed mice, and somewhat lower in untreated mice. Both let-7a and mir-16 are reported to be abundant in liver (Lagos-Quintana *et al.*, 2002). In testis and sperm, mir-16 was higher expressed compared to the other miRNAs, and similar patterns between the three treatment groups were observed. Mir-191 is known to be highly expressed in testis which agrees with other studies (McIver *et al.*, 2012).

Extracting miRNA from sperm cells is challenging because the very small amount is at high risk of being degraded during the extraction procedure. In addition, technical challenges such as low-volume pipetting when preparing the real-time PCR reaction introduces a major source of variation. However, the miRNA results show that it is feasible to detect miRNA from a limited amount of sperm

4.4 Sources of variability

The large standard error observed for genes in sperm samples, may be due to the large variation in the Ct-values which occur when target quantities are approaching single copy, indicated by high Ct-values (34-40) (Applied Biosystems 2011). High Ct-values were detected for mRNAs and miRNAs in sperm samples which indicate the low amount of RNA transcripts in mature sperm cells. Poor pipetting of identical replicates could also cause high standard errors. Per biological replicate, it is common to use three technical replicates, but in order to reduce standard error and increasing the power of the statistical tests, the use of five technical replicates is recommended, in particular when working with low abundance genes.

Other sources of variability which could lead to inaccurate real-time PCR data are air bubbles in the reaction well, PCR mixes not evenly distributed and poor RNA and/or cDNA quality. Uneven contamination from somatic cells during the RNA isolation may also introduce variability of the real-time PCR data. Reducing variability is important if sperm RNAs are to be used as a marker of sperm-quality.

4.5 Conclusions

The overall aim in this study was to examine whether the sperm RNA transcript levels could be used as marker for sperm-quality. We also established method for isolating good quality sperm RNA with minimal contamination of somatic cells. However, a control for a potential somatic cell contamination should be included in further analysis.

Our results indicated that the mRNA transcript levels of the protamine genes, may be used as sperm-quality markers, however, these mRNA transcripts need to be validated in other systems because both BaP- and corn oil-exposure altered their expression in both sperm and testis. Furthermore, the expression of the miRNAa was detected successfully in sperm samples.

Overall, our results indicated that the magnitude of mRNA and miRNA transcripts levels were modest following BaP-exposure, suggesting that the BaP-induced expression went back to baseline at least in liver and testis.

4.6 Future work

A follow-up of this study would be to investigate possible alteration of the protamine ratio at protein levels in sperm and testis after BaP- and corn oil-exposure. To further optimize the sperm RNA isolation procedure, finding primers specific for somatic tissue to identify contaminants in sperm RNA samples would be valuable in order to isolate pure sperm RNA.

Furthermore, the mRNA and especially miRNA expression data needs further validation using additional biological and technical replicates.

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6 Appendix A

6.1 Gene expression results

6.1.1 RNA yield and purity

Table 6-1. RNA yield and purity from sperm cells are shown along with number of sperm cells isolated from each mouse used in the experiment.

Treatment	Sperm cells x 10^6	n	RNA per 1 million sperm cells (µg)	260/280	260/230
BaP	19.9 ± 0.3	10	1.8 ± 0.3	2.0 ± 0.01	1.9 ± 0.11
Corn oil	22.2 ± 0.2	6	1.6 ± 0.2	2.0 ± 0.01	2.0 ± 0.11
Ct	17.4 ± 0.3	4	1.8 ± 0.3	2.0 ± 0.03	1.7 ± 0.16

Cells, RNA yield and purity is shown as mean ± SE. No significantly differences were found between BaP-, corn oil-treated and untreated mice.

Table 6-2. RNA yield and purity from liver and testis with the amount of tissue used per RNA isolation.

Treatment	Mean amount of Tissue (mg)	n	RNA per mg tissue (ug)	260/280	260/230
BaP	27.1 ± 3.9	10	2.4 ± 0.2	2.1 ± 0.02	1.8 ± 0.2
Corn oil	19.1 ± 1.1	6	2.6 ± 0.1	2.0 ± 0.02	2.1 ± 0.2
Ct	23.8 ± 2.4	4	2.5 ± 0.1	2.1 ± 0.01	1.5 ± 0.2

Amount of tissue, RNA yield and purity is shown as mean ± SE. No significantly differences were found between BaP-, corn oil-treated and untreated mice.

6.1.2 cDNA yield and purity

The cDNA yield and purity was calculated and are presented in table 6-1 as mean and standard deviation values. The cDNA yield is much higher from sperm cells, this is due to the addition of carrier RNA.

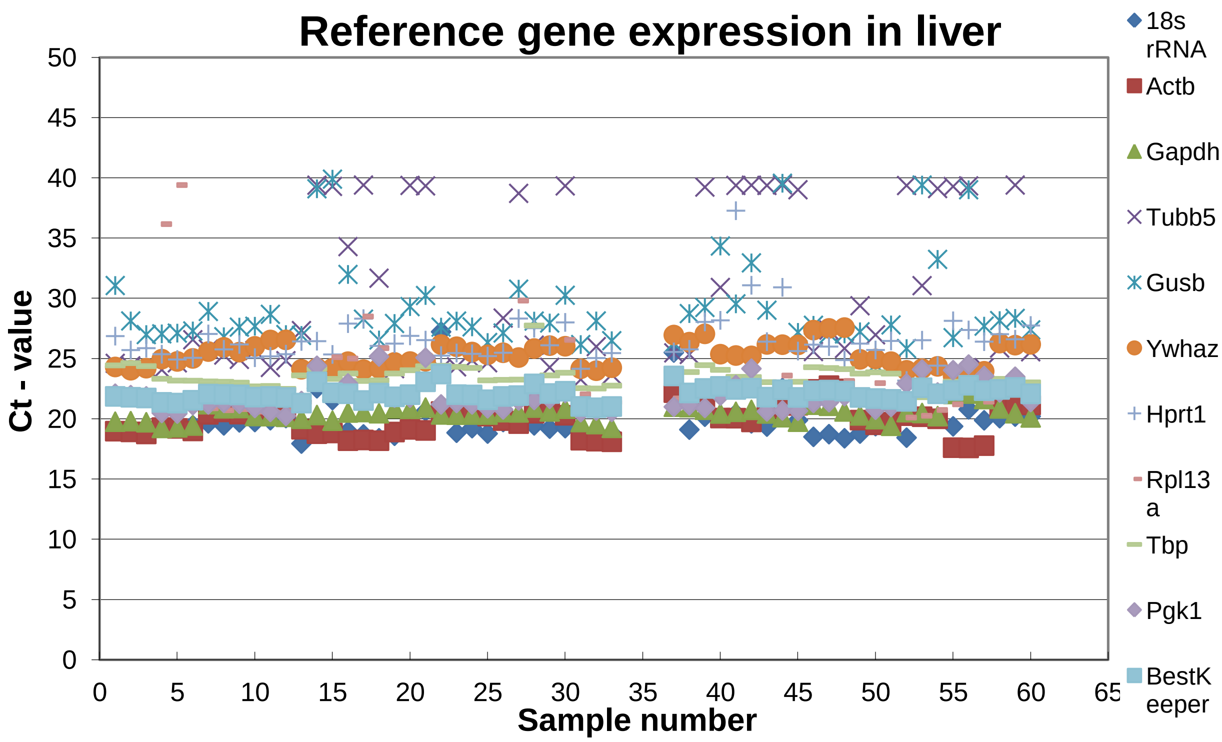
Table6-1. cDNA yield and purity from testis, liver and sperm. The data is presented as mean and standard deviations.

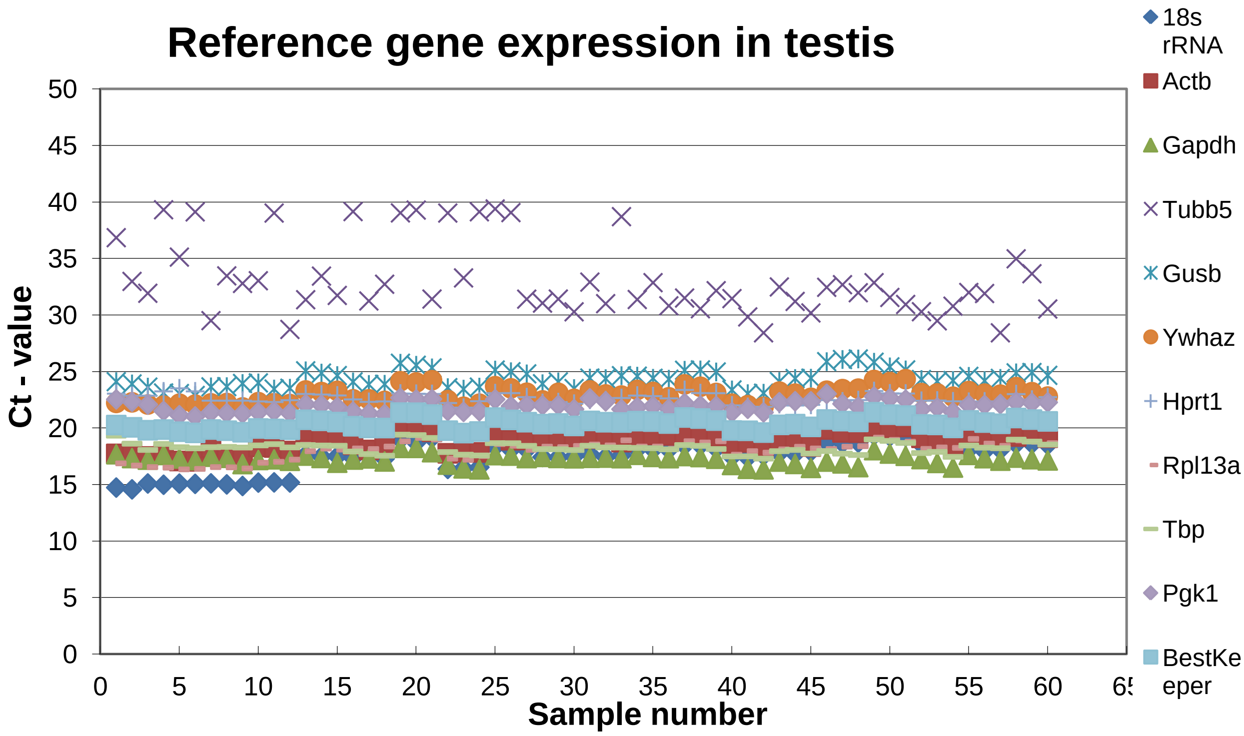
Tissue	n	cDNA (µg)*	260/230	260/280
Testis	20	53.7 ± 6.9	2.2 ± 0.1	1.8 ± 0.03
Liver	20	41.0 ± 4.9	2.3 ± 0.1	1.8 ± 0.02
Sperm	20	142.4 ± 28.9	1.8 ± 0.05	2.2 ± 0.06

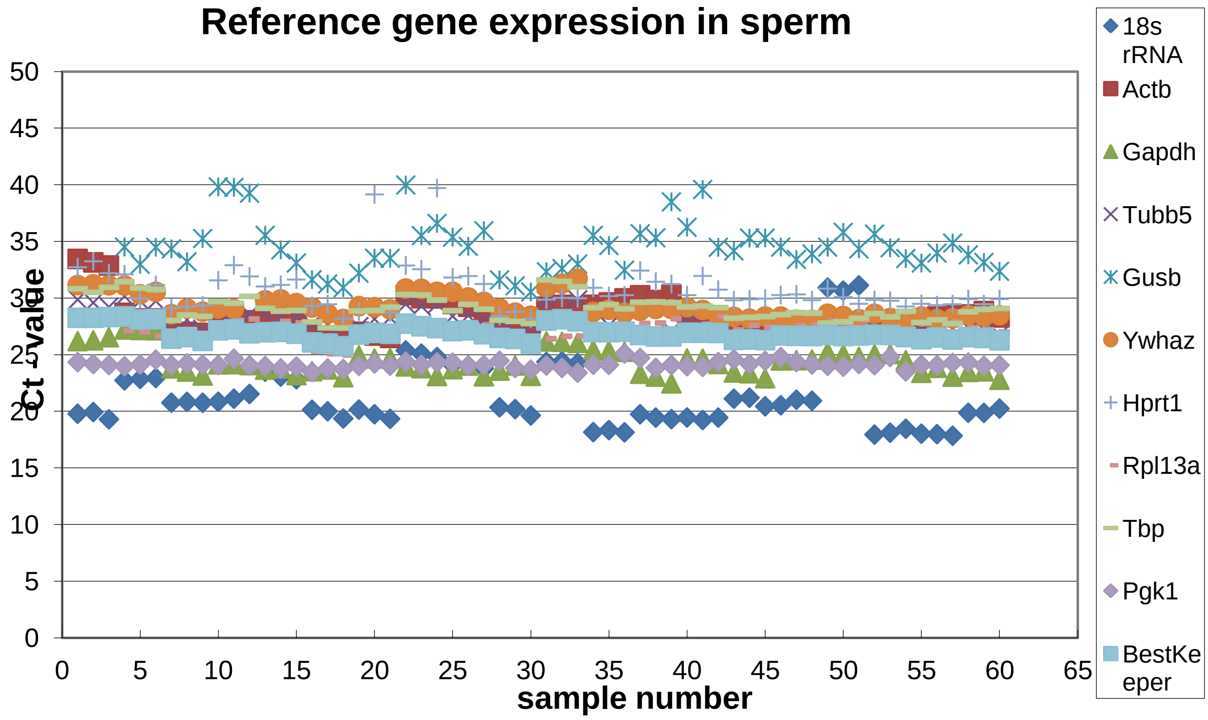
* 1 µg of total RNA was used to convert all RNA samples into cDNA.

6.1.3 Selecting reference genes

Specific reference genes were selected to each type of tissue, using BestKeeper. The expression level for each reference gene are shown below.







6.1.4 ΔΔCt-method

Calculating the relative expression level by using the ΔΔCt method (Livak and Schmittgen2001):

1. The difference in Ct-value for the target gene and the reference gene (ΔCt) was calculated for each sample: $Ct_{\text{target gene}} - Ct_{\text{reference gene}}$
2. Then the ΔCt for the control sample was subtracted from the ΔCt for the treated sample yielding the ΔΔCt: $(Ct_{\text{target gene}} - Ct)_{\text{untreated sample}} - (Ct_{\text{target gene}} - Ct_{\text{reference gene}})_{\text{sample}}$
   The normalized amount relatively compared to the untreated control sample is then: samples is then: $2^{-\Delta\Delta Ct}$ (indicating a 2-fold increase per cycle assuming the efficiency is 100 %). The interpretation of the $2^{-\Delta\Delta Ct}$ value is as follows:
   $2^{-\Delta\Delta Ct} = 1$ No change in gene expression

$2^{-\Delta\Delta Ct} > 1$ Up-regulation of gene expression relatively compared to the control

$2^{-\Delta\Delta Ct} < 1$ Down-regulation of gene expression relatively compared to the control

6.1.5 Log (2) transforming $2^{-\Delta\Delta Ct}$ values

Log_2 transforming data: The reason why the $2^{-\Delta\Delta Ct}$ values are log_2-transformed, is because it becomes easier to compare change in expression between genes. If only ratios are used ($2^{-\Delta\Delta Ct}$ is really a ratio of the normalized value of the treated sample/ normalized value of the untreated control) it becomes difficult to compare directly. For example:

- Increased transcription of 16 = 2^4 which is equivalent to log_2 of 4.
- Repressed transcription of 16 is equal to log_2 of -4 ($1/16 = 16^{-1} = 2^{-4}$).
- By log_2-transforming the ratios 16 and 1/16 it is easier to see the degree to which different genes alter their transcription.

7 Appendix B

7.1 Solutions and media

Homemade real-time PCR master mix for mRNA detection

Components	Volume added (ul)	Final concentration
1M Tris, pH 8.3	100	100 mM
25 mM MgCl	240	6 mM
10 mg/ml BSA	50	1 mg/ml
10 mM dNTP mix	40	200 µM
ddH_2O	530	
SYBR Green	20	0.66 X
ROX	20	
HotStarTaq plus DNA polymerase (5 U/ul)	1,7	0.008 U/µl
Total Volume	**1001,7**	

Hypotonic treatment (SCLB)

SCLB; 0.1 % SDS, 0.5 % Triton X (100 %) solved in deionised water

TE-buffer for dissolving primers

10 mM Tris

1 mM EDTA

Tris and EDTA were dissolved in distilled DNase and RNase free water (UltraPure, Gibco, Invitrogen) and pH was adjusted to 8.0.

7.2 Products and producers

Products	**Producers**
Bap	Sigma
Bürker counting chamber	Labor Optik
Corn oil	Sigma
Distilled water (ddH2O)	NIPH, Norway

Eppendorf tubes 1.5 mL	Sigma
Ethanol (Absolute)	Arcus
Falcon tubes	Nunc
Hepes buffered medium (M2 medium)	Sigma
High-Capacity cDNA Reverse Transcription Kits	Applied biosystems
M2 medium	Sigma
MicroAmp 96-well reaction plate	Applied Biosystems
MiRNeasy kit	Qiagen
miScript Reverse Transcription Kit	Qiagen
miScript SYBR Green PCR kit	Qiagen
Nanodrop 1000	Nanodrop
PBS	Dulbecco
Primers	invitrogen
RNase-Away	Sigma-Aldrich
Ultra-turrax	IKA-Werke
Triton X	Sigma
Ultra turrax	IKA-Werke
Yeast tRNA	Invitrogen

7.3 Primers

Gene symbol	Primer	Description	Sequence	Length	Tm°
Prm1	Left	Protamine 1	GACAGCCCACAAAATTCCAC	20	64.3
	Right		CAGAGCAGGGGACACCAC	18	64.7
Prm2	Left	Protamine 2	GAAGGCGGAGGAGACACTC	19	64.1
	Right		CTCCTCCTTCGGGATCTTCT	20	63.6
Dnmt1	Left	DNA (cytosine-5-)-methyltransferase 1	CAAATAGATCCCCAAGATCCAG	22	63.3
	Right		CGGAACTAGGTGAAGTTTCAAAAA	24	64.1
Crem	Left	cAMP responsive element modulator	TCACAGCAGGATCGAAGTGT	20	63.6
	Right		TGATCCAGCTACAGAAACCTGA	22	63.7
Cyp1a1	Left	Cytochrome P450, family 1, subfamily A, polypeptide 1	TCTTTTGGGAGGAAGTGGAA	20	63.5
	Right		TCCATACATGGAAGGCATGA	20	63.9
Cyp1b1	Left	Cytochrome P450, family 1, subfamily B, polypeptide 1	AGCCAGGACACCCTTTCC	18	64.2
	Right		CCTGAACATCCGGGTATCTG	20	64.2
Tbp	Left	Tata-box binding protein	CGGTCGCGTCATTTTCTC	18	64.5
	Right		GGGTTATCTTCACACACCATGA	22	63.5
Ywhaz	Left	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta	CTTCCTGCAGCCAGAAGC	18	64
	Right		GGTTTCCTCCAATCACTAGCC	21	63.6
Actb	Left	Beta-actin	CTAAGGCCAACCGTGAAAAG	20	63.4
	Right		ACCAGAGGCATACAGGGACA	20	64.6
Tubb5	Left	Tubulin-beta 5	CTGAGTACCAGCAGTACCAGGAT	23	63.3
	Right		CTCTCTGCCTTAGGCCTCCT	20	63.8
Hprt1	Left	Hypoxanthine phosphoribosyltransferase 1	TCCTCCTCAGACCGCTTTT	19	63.9
	Right		CCTGGTTCATCATCGCTAATC	21	63.3
Gapdh	Left	Glyceraldehyde-3-phosphate dehydrogenase	CAGCAAGGACACTGAGCAAGAG	22	66.4
	Right		GCCCCTCCTGTTATTATGGGGGTC	24	71.5
18S rRNA	Left	18S ribosomal RNA	GGATTTGTGCCGGGGACGGA	20	75.3
	Right		CCCGTCGGGGTCCGACAAAAC	21	75.3
Gusb	Left	Beta-glucuronidase	CTCTGGTGGCCTTACCTGAT	20	63
	Right		CAGTTGTTGTCACCTTCACCTC	22	63.6
Pgk1	Left	Phosphoglycerate kinase 1	TACCTGCTGGCTGGATGG	18	65
	Right		CACAGCCTCGGCATATTTTCT	20	64
Rpl13a	Left	Ribosomal protein L13a	ATCCCTCCACCCTATGACAA	20	63.1

	Right		GCCCCAGGTAAGCAAACTT	19	62.9
Apex1	Left	APEX nuclease (multifunctional DNA repair enzyme) 1	CGGGGAAGAACCCAAGTC	18	60
	Right		TCCTTCTCGGTTTTCTTTGC	20	59
Ogg1	Left	8-oxoguanine DNA glycosylase	TTATCATGGCTTCCCAAACC	20	59
	Right		GGCCCAACTTCCTCAGGT	18	60
Neil1	Left	Endonuclease VIII-like 1	TCGTAGACATCCGTCGCTTT	20	60
	Right		TGTCTGATAGGTTCCGAAGTACG	23	60
Xpc	Left	Xeroderma pigmentosum, complementation group C	CCTATCGGAGCCTACTTACGG	21	60
	Right		AGGTGCTTCGCCTGAAACT	19	60
Xrcc1	Left	X-ray repair cross-complementing protein 1	GCCTTTGTGGAGGTGCTAGT	20	59
	Right		AAAGAGGAGGTGACCAGAAGG	21	59